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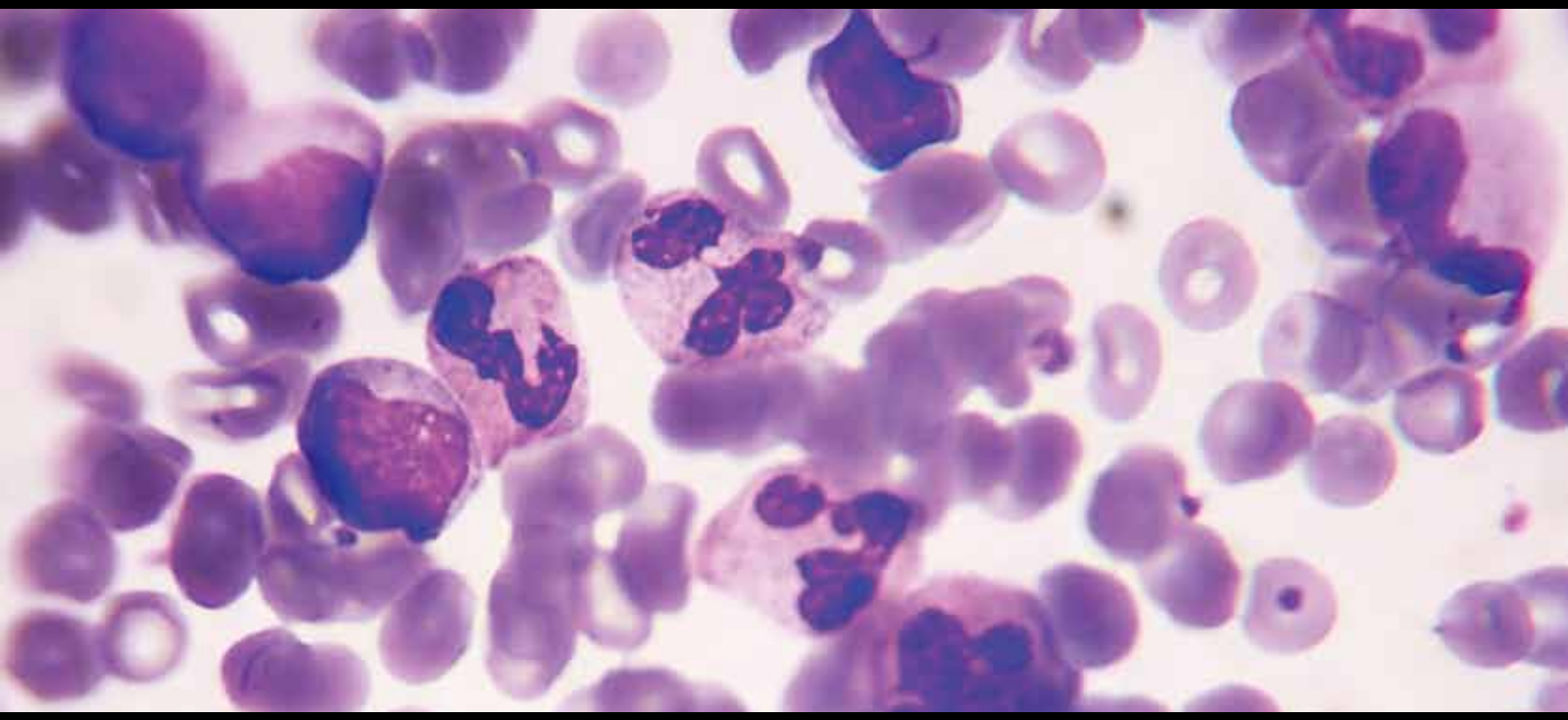
Pathology of Breast Carcinoma: Diagnostic, Prognostic, and Therapeutic Issues and Challenges

Guest Editors: Rohit Bhargava, Sunati Sahoo, Nicole Nicosia Esposito, and Beiyun Chen



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Editorial Board

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Editorial

Pathology of Breast Carcinoma: Diagnostic, Prognostic, and Therapeutic Issues and Challenges

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Worldwide, breast cancer is the most common cancer affecting women. The disease is more common in the affluent world, but breast cancer incidence is steadily rising in the developing world. Breast cancer is a heterogeneous disease morphologically, immunohistochemically, and also at the molecular level regardless of the women's race or geographic location. Therefore, the prognosis and response to standard treatments can be highly variable from one patient to another. The first step towards personalized therapy for breast cancer is to understand the heterogeneity of the disease. Pathologists have been aware of breast cancer heterogeneity for a long time and, therefore, devised a grading system to identify tumors with different prognoses. Breast cancer grading is still very useful clinically (as illustrated in one of the original manuscripts in this issue by M. Purdom et al.), but additional prognostic/predictive markers have further improved our ability to personalize breast cancer therapy. The division of breast tumors into estrogen receptor (ER) positive and estrogen receptor-negative groups was an important step in recognizing heterogeneity and use of antiestrogen therapy in ER-positive disease is the first example of targeted therapy in human cancers. Recent advances in gene expression profiling have further exemplified the heterogeneity of breast cancer. The ER-positive disease is now split into at least 2 groups, one with good prognosis (likely luminal A type tumors) and another with poor prognosis (likely luminal B type tumors). The ER-negative disease is also split into an HER2-enriched category and the so-called basal-like breast cancer category. Although the molecular classes described using gene expression profiling do have morphologic and immunohistochemical correlates, there is lack of consensus on a defined morphologic or immunohistochemical criteria. Nevertheless, the molecular and subsequent immunohistochemical studies provided a framework for studying various other parameters or novel biological markers with respect to the best known prognostic/predictive markers ER, progesterone receptor (PR), and HER2. Two original articles in this issue have used similar immunohistochemical definitions to address the issue of multifocality in breast cancer (T. Tot et al.) and to study the prevalence of basal-like breast cancer in Sudan (K. D. Awadelkarim et al.).

The recent advances in breast cancer research has increased our understanding of breast cancer and provided us with more refined classifications. However, classifications do not immediately help breast cancer patients, but the hope is that understanding the molecular network will pave way for more targeted treatments. Therefore, studies of novel breast cancer markers are always useful. One review article and two original articles addressing these issues are included in the current issue (review by B. Uziely at al. and original articles by Y. Y. Wang et al. and by A. Nasir et al.).

In addition to the study of novel markers, traditional issues related to breast cancer are also discussed in this issue. Despite the identification of novel markers that provides insight into breast tumor biology, lymph-node metastasis still remains one of most important prognostic factor in breast cancer. V. Van Belle et al. report on the issue of ratio of positive versus negative lymph nodes by performing a comparative study of nottingham prognostic index (NPI) versus log odds prognostic index (Lpi) for short-term breast cancer-specific disease free survival. Two review articles

(one by P. Tai et al. and another by G. Peclivanides et al.) discuss the issues related to sentinel lymph node biopsy and assessment.

Another common, but important issue in breast cancer is assessment of *HER2* (*ERBB2*) oncogene. *HER2* gene is amplified/overexpressed in approximately 20% of breast cancer and is a marker of aggressive disease. However, with the availability of HER2-targeted therapy, trastuzumab, the natural history of HER2 disease has been significantly altered. Trastuzumab treatment is highly effective in HER2 positive tumor, but it is generally ineffective in HER2-negative disease. Moreover, the treatment is expensive and potentially cardiotoxic, so patient selection is very important. Due to the availability of such an effective treatment, it is very important for pathologists to be aware of all HER2-testing issues. The article on HER2 testing in breast cancer by S. Shah and B. Chen provides a comprehensive review on the subject.

Other review articles included in this issue update the reader on less well-known but increasing-talked-about topics in recent months, such as angiogenesis in breast cancer (E. S. Ch'ng et al.), circulating tumor cells in breast cancer (E. S. Graves et al.), and breast cancer metastasis to the brain (F. Arshad et al.). Treatment of breast cancer metastasis is discussed by A. M. Stark.

The scope of breast cancer research is quite broad, and, is difficult to include all topics in one issue. Nevertheless, we have tried to include both common and uncommon topics in a mix of high-quality original and review articles to satisfy the need of readers with different backgrounds. We hope the readers will find this special issue on breast cancer stimulating.

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Research Article

Should Histologic Grade Be Incorporated into the TNM Classification System for Small (T1, T2) Node-Negative Breast Adenocarcinomas?

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Prognosis of invasive ductal carcinoma (IDC) strongly correlates with tumor grade as determined by Nottingham combined histologic grade. While reporting grade as low grade/favorable (G1), intermediate grade/moderately favorable (G2), and high grade/unfavorable (G3) is recommended by American Joint Committee on Cancer (AJCC) staging system, existing TNM (Primary Tumor/Regional Lymph Nodes/Distant Metastasis) classification does not directly incorporate these data. For large tumors (T3, T4), significance of histologic grade may be clinically moot as those are nearly always candidates for adjuvant therapy. However, for small (T1, T2) node-negative (N0) tumors, grade may be clinically relevant in influencing treatment decisions, but data on outcomes are sparse and controversial. This retrospective study analyzes clinical outcome in patients with small N0 IDC on the basis of tumor grade. Our results suggest that the grade does not impact clinical outcome in T1N0 tumors. In T2N0 tumors, however, it might be prognostically significant and relevant in influencing decisions regarding the need for additional adjuvant therapy and optimal management.

1. Introduction

While the World Health Organization, College of American Pathologist, and American Joint Commission on Cancer all endorse reporting histologic tumor grade for IDC, it does not directly factor into the current TNM staging system [1–3]. The Nottingham Combined Histologic Grade (NCHG), the preferred grading system, stratifies tumors into three grades based on semiquantitative evaluation of tubule formation, nuclear pleomorphism, and mitoses [4]. Histologic tumor grade, as determined by NCHG, correlates with prognosis [5] and might represent a simple and inexpensive way to identify low-risk patients who are highly curable by surgery alone or are also in need of adjuvant therapy [6, 7]. Patients with large tumors are almost always candidates for adjuvant therapy, so incorporating histologic grade in such cases may be clinically irrelevant [8]. Also, tumor size often correlates with tumor grade [9]. In this era of mammographic screening, however, an increasing proportion of identified breast cancers are small and node negative. Whether or not histologic grade is an independent prognostic factor in small, node-negative IDC is still an unresolved question [10]. The Breast Task Force of the AJCC has noted that the data on this issue are sparse and inconsistent, and as such, it refrained from directly including the histologic tumor grade into the TNM staging. While the existing data clearly differentiate the prognosis of G1 and G3 tumors, the behavior of G2 tumors is ambiguous owing to methodologic differences (followup times, grading systems, and measured outcomes). We undertook a retrospective study to analyze the clinical

Table 1: Tumor stage, grade, and clinical outcome.

	T1a ($n = 10$)			T1b ($n = 23$)			T1c ($n = 45$)			T2 ($n = 33$)		
	G1	G2	G3	G1	G2	G3	G1	G2	G3	G1	G2	G3
Alive without disease	1	4	3	5	10	5	8	20	15	1	15	11
Alive, status unknown	—	—	1	—	1	—	—	—	—	—	—	—
Alive with disease	—	1	—	—	—	—	—	—	—	—	—	1
Deceased without disease	—	—	—	—	1	—	—	1	1	—	1	1
Deceased, cause unknown	—	—	—	—	—	—	—	—	—	—	—	—
Deceased with disease	—	—	—	1	—	—	—	—	—	—	—	3
Total	1	5	4	6	12	5	8	21	16	1	16	16

G—tumor histologic grade.

Table 2: Clinical outcome by tumor grade in small (T1, T2) node-negative breast adenocarcinomas.

Tumor Size	T1 ($N = 76$)*					T2 ($N = 33$)				
	*Clinical Outcome***					*Clinical Outcome***				
	Without Disease		*With Disease*			*Without Disease*		*With Disease*		
Tumor Grade	*N*	%	*N*	%	*P****	*N*	%	*N*	%	*P****
G1-G2	50	66	2	3		17	52	0	0	
G3	24	31	0	0	.46	12	36	4	12	.04

*Excludes 2 alive, status unknown.
**Clinical outcome includes patients living and deceased.
***The p-value is for a one-tailed Fisher exact test.

outcomes in patients with small, node-negative cancers in an attempt to contribute to this ongoing debate regarding the prognostic significance of histologic tumor grade.

2. Design

The files of the Department of Pathology, University of Kentucky Medical Center were searched from January 1995 through July 2007 and yielded a total of 111 lumpectomy/mastectomy specimens from patients with T1N0 or T2N0 tumor status. The cases included 10 T1a, 23 T1b, 45 T1c, and 33 T2 tumors. The age of patients ranged from 31 to 83 years (mean, 55 years). The length of followup ranged from 7 to 152 months (mean, 56 months), with at least 60 months (5 years) and longer followup in 56% of patients. Presence of coexistent ductal carcinoma in-situ (DCIS), lymphovascular invasion (LVI), estrogen and progesterone receptor (ER/PR), and HER-2/neu status by immunohistochemistry was analyzed. Clinical followup data with outcome through year 2008 were obtained from Tumor Registry. The tumor size and histologic grade in conjunction with clinical outcome was analyzed.

3. Results

The data for tumor size, histologic grade, and patient status are summarized in Table 1. On the followup of patients with T1 tumors, 71/78 (93%) were alive and 3 deceased without disease. Only one patient died with disease (G1/stage T1b), and another patient was alive with disease recurrence (G2/stage T1a). Two patients were alive with status unknown. Of the patients with T2 tumors, 27/33 (82%) were alive and two deceased without disease on followup. Three died with disease (all ER negative, including one triple negative), and one was alive with disease; all four (12%) had G3 tumors (including two with LVI). All patients with hormone receptor positive tumor status received Tamoxifen or aromatase inhibitors. Of patients with T1 tumors, 24% received chemotherapy, as did 67% of patients with T2 tumors (2 of 4 with recurrent/ progressive disease had chemotherapy).

Of T1 tumors, 76% (59/78) showed DCIS versus 69% (22/32) in T2 tumors. LVI was identified in 3% (1/33) of T1a/T1b, 13% (6/45) of T1c, and 24% (8/33) of T2 tumors. Positive ER and PR status was reported in 71% (55/78) and 64% (50/78) of T1 tumors, respectively, and 63% (20/32) and 69% (22/32) of T2 tumors, respectively. All three deceased patients with T2 tumors were tested ER negative. Two of those three tumors also showed LVI. The patient with the T1b tumor who died of disease had ER-positive tumor and no LVI. HER-2/neu status was unknown in this case. Positive HER-2/neu status was reported in 1/61 T1 tumors and 5/26 T2 tumors. Of three deceased cases, HER-2/neu status was reported in only one and was negative. Both patients who are alive with disease had positive ER and negative HER-2/neu tumors, and no LVI.

The relationship between the tumor grade and clinical outcome moderated by tumor size has been determined by using the Fisher exact tests (Table 2).

The results of the Fisher exact tests suggest an interaction effect—with the relationship between tumor grade and clinical outcome moderated by tumor size. That is, among patients with T1 tumors ($n = 76$), clinical outcome did

not vary significantly by tumor grade ($P = .46$). However, among the T2 group ($n = 33$), the number of patients with G3 tumors who were with disease ($n = 4$) was significantly greater than those with G1-G2 tumors ($n = 0$)($P = .04$). The strength of the relationships (φ) between tumor grade and clinical outcome for the T1 and T2 groups was.11 and.38, respectively.

4. Discussion

Regardless of histologic grade, the overall prognosis for small node-negative breast adenocarcinomas appears to be very good. In the current study, the disease-free survival for patients with T1N0 tumor status was 93% (71/76) and for patients with T2N0 was 88% (29/33). This relatively good prognosis is similar to that reported in prior studies [5, 11, 12], and the difficulty of addressing the prognostic significance of histologic grade in these breast cancers is highlighted. Namely, studies would need to be larger to have the statistical power to detect a relationship between histologic grade and clinical outcomes. Beyond sample size, length of followup is also an important consideration as recurrence may occur quite late [13–15].

While some studies have shown histologic grade to be prognostically significant in small, node-negative breast carcinomas [5, 16, 17], other studies [18–20] do not demonstrate this association. Lundin et al. suggest that omission of histologic grading from clinical decision making may result in considerable overuse of adjuvant therapies [21]. Based on our data in this relatively small study, there is no evidence that higher tumor grade impacts the clinical outcome in T1N0 tumors. This tumor status was not associated with higher rate of recurrence or disease persistence in our study. In T2 tumors, however, our data suggest that histologic tumor grade might be prognostically significant and relevant in influencing decisions regarding the need for additional adjuvant therapy and optimal management of node-negative breast carcinomas at this stage.

Currently, adjuvant hormonal and/or cytotoxic chemotherapies are recommended for most women with early-stage invasive breast cancer. Treatment decisions are based on axillary node status, age, tumor size, histologic tumor type, tumor grade, hormone receptor status, and coexisting medical conditions [22]. However, most patients with node-negative disease who receive chemotherapy will not derive benefit because they would not go on to have a recurrence even without such treatment, which also questions the necessity of performing the Oncotype Dx testing in T1N0 tumors. New prognostic and predictive tests are needed to better individualize therapy and confine systemic treatment, especially cytotoxic chemotherapy, to those patients who are most likely to benefit [23, 24]. Although based on a limited material, our data may suggest more favorable prognosis for patients with T1N0 regardless of tumor grade, as well as low-grade T2N0 tumors, compared to those with high-grade T2N0 disease who might benefit from additional chemotherapy. Larger studies with considerable statistical power will be needed to definitively demonstrate the impact of histologic grade in these subsets of breast adenocarcinoma.

Several relatively recent studies indicate that the histologic tumor grade appears to reflect specific molecular predictive indicators such as proliferative markers and multigene expression arrays [25, 26]. Interestingly, the grading was shown to correlate with other proposed prognostic factors such as Recurrence Score (Oncotype Dx) and casting-type microcalcifications [23, 24, 27]. In the Kaiser population, tumor size and tumor grade remained statistically significant associated with the risk of breast cancer death in most multivariate models that also included the Recurrence Score [23], whereas only tumor grade remained independently associated with risk in the NCABP B-14 study [28]. The Recurrence Score was able to identify a larger subset of patients with low risk of breast cancer death than was possible with either of the standard prognostic indicators [23].

While currently the Breast Cancer Task Force has elected not to include histologic grade as a stage-modifying factor in the TNM system [8], it still does recommend collection of tumor grade, using the standardized Nottingham combined histologic score with calibrated mitotic counts, for inclusion in tumor registry database [1]. How to merge histopathologic data with clinical, radiographic, and molecular information into a therapeutic plan is an evolving challenge. While many studies indicate the significance of Recurrence Score in predicting the magnitude of chemotherapy benefit, given the financial constraints and limited access to molecular testing within many health care systems, studying the utility of histologic grade (along with other parameters) continues to be relevant. For example, based on the literature as well as our data, it might appear that the Oncotype Dx testing (quoted price $4,075 per test) is adding little or no additional prognostic value to T1N0 and low-grade T2N0 tumors, which almost always show favorable outcome with no recurrence. Ultimately, determining if histologic grade will independently provide clinically relevant information in these cases to serve as a decision tool in the adjuvant chemotherapy setting merits further investigation with a large data set, extended followup, and standardized reporting.

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Research Article

Molecular Phenotypes of Unifocal, Multifocal, and Diffuse Invasive Breast Carcinomas

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We analyzed the subgross distribution of the invasive component in 875 consecutive cases of breast carcinomas using large-format histology sections and compared the immunophenotype (estrogen and progesterone receptor expression, HER2 overexpression and expression of basal-like markers, CK5/6, CK14, and epidermal growth factor receptor) in unifocal, multifocal, and diffuse tumors. Histology grade and lymph node status were also analyzed. Unifocal invasive carcinomas comprised 58.6% (513/875), multifocal invasive carcinomas 36.5% (319/875), and diffuse invasive carcinomas 4.9% (43/875) of the cases. The proportion of lymph node-positive cases was significantly higher in multifocal and diffuse carcinomas compared to unifocal cancers, but no other statistically significant differences could be verified between these tumor categories. Histological multifocality and diffuse distribution of the invasive tumor component seem to be negative morphologic prognostic parameters in breast carcinomas, independent of the molecular phenotype.

1. Introduction

Breast carcinoma is a heterogeneous group of diseases; individual cases deviate from each other in morphology, phenotype, and prognosis. Using DNA microarray technique and cluster analysis, five distinct genetic types of the disease were delineated: luminal A, luminal B, HER2 positive, basal-like, and normal breast like tumors [1, 2]. These tumor subtypes can also be identified with sufficient accuracy during routine diagnosis, using a simple panel of immunohistochemical markers, including antibodies tracing estrogen receptors (ER), progesterone receptors (PR), c-erbB-2 oncoprotein overexpression (HER2), and some myoepithelial markers [3, 4]. The recommended myoepithelial markers for delineating the basal-like tumors vary in different studies, cytokeratin (CK)5/6, CK14, CK17, and/or epidermal growth factor receptor (EGFR) being used most often [3, 5–8]. Significant differences in survival of patients with different molecular subtypes of breast carcinoma have been recently evidenced; Luminal A tumors have a significantly better 5- and 10-year survival compared to luminal B, HER2 positive, basal-like, and unclassified tumors [9].

Using large-format histologic sections in diagnostic routine, we have repeatedly evidenced that breast carcinoma has a complex subgross morphology with a considerable proportion of the tumors being either multifocal or diffuse [10–13]. The most recent studies on breast cancer multifocality indicate that multifocality and diffuse distribution of the invasive tumor component represent survival-related negative prognostic parameters [14–17]. As we have not found corresponding data in the literature, we designed the present study with the aim to analyze the relation of subgross appearance of the lesions (unifocal, multifocal, or diffuse distribution of the invasive component) and some phenotypic tumor features, such as ER, PR, and HER2 expression, basal-like phenotype, and histology grade. We focused on the invasive component of the tumor and did not analyze the distribution of the in situ ductal or lobular

components in this study. We also tested the relation of the subgross histologic distribution of the lesions to presence of lymph node metastasis (LNM).

2. Materials and Methods

2.1. Study Population. This study is a retrospective analysis of 875 consecutive breast carcinoma cases diagnosed at the Department of Pathology and Clinical Cytology of the Central Hospital in Falun, Sweden, during the period January 2005−December 2009. Patients with recurrent breast carcinomas that were diagnosed before the study period were excluded. We also excluded purely in situ carcinomas (132 cases), microinvasive (<1 mm) carcinomas, and carcinomas which were not routinely stained for the immunohistochemical markers listed below. The subgross parameters, histology grade, LNM, ER, PR, and HER2 status were analyzed during the entire study period. The basal-like phenotype was routinely assessed from September 2006 to the end of the study period. The study was approved by the Regional Ethical Committee in Uppsala-Örebro region.

2.2. Large-Section Histopathology. All specimens were prepared by the method of large-section histopathology, which has been a routine procedure in our laboratory since 1982. The method has been described in detail elsewhere [18]. Briefly, all cases were discussed by a preoperative tumor board, and the radiological (mammography, ultrasound, and magnetic resonance imaging) appearance was registered, including the extent and distribution. This information, together with the whole-specimen radiograph received with the surgical specimen, guided the pathologist during the workup. The sector-resection specimens were sliced into 3-4 mm-thick tissue slices parallel to the pectoralis fascia. The slices were also radiographed. One to five of the most representative slices (measuring up to 9×8 cm) were selected for embedding into large paraffin blocks. Larger slices were bisected and embedded into separate blocks. Mastectomy specimens were sliced perpendicular to the pectoralis fascia to visualize the surgical margin in one histological plane. All cases were discussed again on postoperative tumor board to check the concordance of the radiological and histological findings. Most cases which were discrepant in favour of radiology findings were solved with additional sampling of the specimen for histological analysis.

2.3. Immunohistochemistry. The largest invasive tumor focus was sampled for routine immunostaining. The following antibodies were used: ER (Ventana Medical Systems, clone : SP1, 1 : 200), PR (Dako, clone : PgR 636, 1 : 50), CK5/6 (Dako, clone : D5/16 B4, 1 : 100), CK14 (Novocastra, clone : LL002, 1 : 20), EGFR (Dako, clone : E 30, 1 : 25), and HER2 (Dako, code A 0485, 1 : 250). Additional foci were only stained in selected cases. Nuclear staining >10% of the tumor cells were the criterion of ER and PR positivity, cytoplasmic staining in >10% for CK5/6 and CK14 positivity, and membranous and cytoplasmic staining in >10% of the tumor cells for EGFR positivity. HER2 positivity was assessed in accordance with the criteria of the manufacturer; all 2+ equivocal cases underwent fluorescence in situ hybridisation test.

2.4. Diagnostic Criteria. The distributions of the invasive component and of the in situ component of the same lesion were determined separately. For the purpose of the present study, the tumors were classified based on the distribution of the invasive lesions. They were considered to be "unifocal" if only one invasive focus could be observed in the large sections, with the tumor focus containing or not containing an in situ component. "Multifocal" invasive lesions were characterized by the presence of multiple well-delineated invasive tumor foci separated from each other by uninvolved breast tissue, regardless of the distance between the foci. Tumors that were dispersed over a large area in the section, much like a spider's web, with no distinct tumor mass were classified as "diffuse." The size of the diffuse tumors was equal to the extent of the disease in many cases and was rather comparable to the extent of the disease in multifocal cases than to the size of the individual foci. When the distribution of the lesions was assessed, in each case, an attempt was made to summarize the findings in different levels of the large sections to reconstruct the in vivo situation before operation. Detailed correlation between radiological and pathological findings was essential. If a secondary surgical intervention was performed in addition to the primary sector resection, an attempt was made to summarize the findings in the entire excised tissue. However, sector resection specimens (average size of 9×6 cm) were sufficient for categorizing the findings in most cases. Typical cases of unifocal, multifocal, and diffuse invasive breast carcinomas are illustrated in Figure 1.

LNM was defined as presence of metastatic deposit(s) in at least one of the lymph nodes of the case, irrespective on the size of the deposit(s). Both sentinel and nonsentinel nodes were assessed on routinely stained sections. The sentinel lymph nodes were additionally stained on CK8/18 (BD Biosciences, clone Cam 5.2, 1 : 50). Tumors expressing at least one of the basal (myoepithelial) markers (CK5/6, CK14, EGFR) in at least one of the invasive tumor foci were categorized as basal-like tumors. Triple-negative tumors were defined as negative for all of the following markers: ER, PR, and HER2. The tumors were graded according to the Notthingham (Bloom-Richardson-Elston-Ellis) grading system [19]. Tumor size was defined as the largest dimension of the largest invasive focus.

2.5. Study Execution. All of the large histological sections belonging to this series (average number of sections per case 6, range of 1–34) were reviewed for the purposes of postoperative tumor board. Histological data, including the distribution of lesions, were determined according to the diagnostic criteria described above and registered in a database. "Multicentricity," which is defined as the presence of malignant structures in different quadrants of the same breast, was not analyzed because it represents a clinical and/or radiological parameter. Phenotypic parameters were obtained from the department's database. The statistical analysis (comparison of proportion using chi-square test)

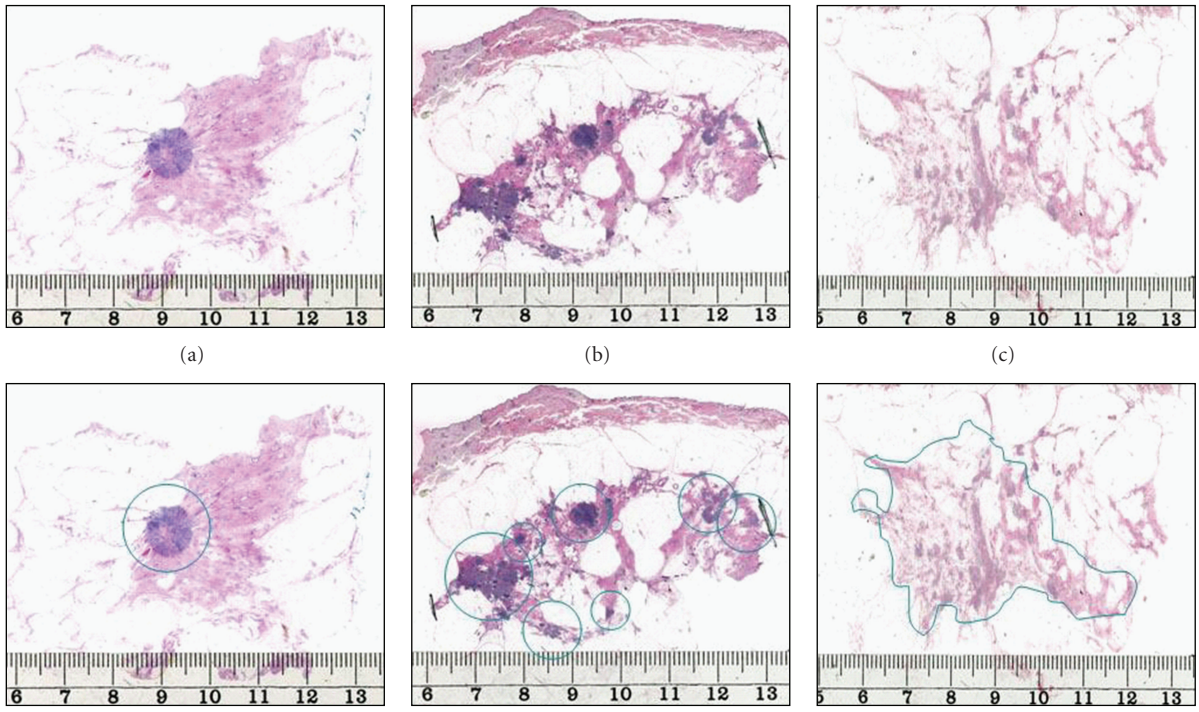

Figure 1: Typical cases of unifocal (left), multifocal (central), and diffuse (right) invasive breast carcinomas documented in large-format histology sections. The lesions are marked in the lower images.

Table 1: Immunophenotypic parameters, histology grade and node status by invasive tumor distribution (unifocal, multifocal, and diffuse) in 875 consecutive breast cancer cases, Falun 2005–2009.

Phenotype	Invasive tumor distribution				Significance level	
	Unifocal	Multifocal	Diffuse	Total	Unifocal versus multifocal	Unifocal versus diffuse
ER positive	83.1% (423/509)	81.8% (256/313)	97.7% (42/43)	83.4% (721/865)	$P = .7852$	$P = .0178$
PR positive	66.3% (331/499)	64.9% (203/313)	53.5% (23/43)	65.1% (557/855)	$P = .8289$	$P = .1565$
HER2 positive	9.6% (48/501)	15.2% (48/315)	4.8% (2/42)	11.4% (98/858)	$P = .0419$	$P = .4334$
Triple negative	11.2% (56/499)	10.5% (33/313)	2.4% (1/42)	10.5% (90/854)	$P = .9083$	$P = .1144$
Basal-like	12.5% (37/296)	11.3% (22/195)	3.7% (1/27)	11.6% (60/518)	$P = .6020$	$P = .2896$
Grade 3	22.6% (115/509)	25.2% (80/318)	7.0% (3/43)	22.8% (198/870)	$P = .5664$	$P = .0245$
Node positive	27.3% (140/513)	54.2% (173/319)	55.8% (24/43)	38.5% (337/875)	$P < .0001$	$P = .0001$

was carried out using commercially available software (MedCalc statistics for biomedical research, MedCalc Software, Belgium), with P-values <.01 regarded significant.

3. Results

The distribution of the invasive lesions could be accurately analyzed in 875 invasive cases. Unifocal invasive carcinomas comprised 58.6% (513/875), multifocal invasive carcinomas 36.5% (319/875), and diffuse invasive carcinomas 4.9% (43/875) of the cases.

Histology grade was determined in 870 of the invasive carcinomas of which 22.8% (198/870) were grade 3. As demonstrated in Table 1, there were 115 unifocal, 80 multifocal, and 3 diffuse grade 3 invasive carcinomas. The percentage of unifocal and multifocal grade 3 cases were very similar (22.6% versus 25.2%). The proportion of grade 3 cases among diffuse invasive carcinomas was only 7.0%, but the difference was not statistically significant as only 3 such cases were found.

Tumor size could be accurately assessed in 511 unifocal (average size 16.0 mm, range 3–70 mm), 315 multifocal (average size 19.5 mm, range 2–60 mm), and in 41 diffuse (average size 45.6 mm, range 20–85 mm) invasive carcinomas. This difference was not statistically significant when unifocal and multifocal tumors were compared ($P = .7375$), but the differences became significant when the diffuse group was added to analysis ($P = .0003$).

ER status was assessed in 865 invasive breast carcinomas. There were 83.4% (721/865) ER-positive and 16.6%

(144/865) ER-negative cases. There were 83.1% (423/509) unifocal, 81.8% (256/313) multifocal, and 97.7% (42/43) diffuse ER-positive invasive cancers. The differences were statistically not significant.

PR was assessed in 855 cases. There were 65.1% (557/855) PR-positive and 34.9% (298/855) PR-negative cases. 66.3% (331/499) of the unifocal, 64.9% (203/313) of the multifocal, and 53.5% (23/43) of the diffuse invasive carcinomas were PR positive. The differences were statistically not significant.

During the study period, 858 invasive breast carcinomas were tested for HER2 overexpression and 11.4% (98/858) were found to be HER2 positive: 9.6% (48/501) unifocal, 15.2% (48/315) multifocal, and 4.8% (2/42) diffuse tumors. The differences were statistically not significant.

The proportion of triple negative cases was 10.5% (90/854) in the present series, 11.2% (56/499) among the unifocal, 10.5% (33/313) among the multifocal, and 2.4% (1/42) among the diffuse cases. The differences were statistically not significant.

Carcinomas were routinely stained for basal markers in 518 cases. Of those, 11.6% (60/518) expressed basal-like phenotype, 12.5% (37/296) of the unifocal, 11.3% (22/195) of the multifocal, and 3.7% (1/27) of the diffuse tumors. The differences were statistically not significant.

LNM was determined in all cases of the present series of invasive breast carcinomas and 38.5% (337/875) of the cases had some form of metastatic tumor spread (including macrometastases, micrometastases, and isolated cancer cells/cellgroups). The proportion of lymph node positive cases was 27.3% (140/513) in the group of unifocal cancers, 54.2% (173/319) in the group of multifocal cancers, and 55.8% (24/43) in the group of diffuse tumors. These differences were statistically highly significant.

4. Discussion

Breast cancer is a disease with wide variation in subgross morphology. Tumor multifocality has been evidenced in a substantial proportion of the cases in early whole organ studies and is seen in >30% in the series of cases documented with large-format histology slides [10–13]. In addition, 5% of the invasive carcinomas exhibit a diffuse, spider's web-like growth pattern [14]. Modern radiology methods, especially if used in combination (multimodality approach), are able to indicate the growth pattern of the tumor in the vast majority of the cases [18]. Detailed radiological-pathological correlation and regular use of large-format histology slides enables the breast pathologist to correctly assess tumor size, disease extent, and multifocality or diffuse growth and to confirm or correct the preoperative radiological results.

The prognostic significance of tumor multifocality has recently received special attention as, in contrast to some previous publications [20], recent long-term followup studies have demonstrated significantly lower breast cancer-specific survival in multifocal than in unifocal tumors [15–17]. Multifocality seems to be a negative morphologic parameter independent of treatment modalities [16]. Diffuse invasive carcinomas have an even worse prognosis than the multifocal tumors [14, 17]. The question arises whether differences in survival between unifocal, multifocal, and diffuse breast carcinomas can be explained with differences in their molecular phenotype. The present study was carried out on a recent series of cases, thus survival of the patients could not be tested.

The greater metastatic capacity of multifocal and diffuse tumors compared to the unifocal ones has been repeatedly proven in independent studies [10–12, 20–22] and was also confirmed in the present study. In fact, this was the only statistically significant difference between unifocal versus multifocal and unifocal versus diffuse invasive carcinomas in the present series.

The relation of tumor multifocality and tumor phenotype is rarely studied in the literature. Histology grade, ER, PR, and HER2 status represent well-established, routinely assessed morphological prognostic parameters [23, 24]. According to our results, no significant differences could be demonstrated between these tumor categories with respect to histology grade, ER, PR, and HER2 status. The same finding has been reported by Litton et al., but their study was limited to women ≤35 years [25]. In the study of Oh et al., no significant differences were found between unifocal and multifocal/multicentric tumors regarding nuclear grade and ER status [26].

While multifocal breast carcinomas had a tendency to show more unfavourable phenotype, although statistically not significantly different, compared to the unifocal tumors, the diffuse invasive carcinomas exhibited an opposite tendency. They were less often ER negative, less often triple negative, less often HER2 positive, or basal-like than the unifocal cancers. Although this is a remarkable phenomenon as these tumors have the less favourable outcome, this may be explained by the high percentage of invasive lobular carcinomas in this subgroup [10, 14].

5. Conclusion

Although multifocal and diffuse invasive breast carcinomas exhibited a doubled frequency of LNM compared to that in unifocal tumors, no statistically significant differences could be demonstrated between these categories regarding histology grade, ER, PR, or HER2 status and regarding the proportion of tumors with basal-like phenotype. Multifocal and diffuse distribution of the invasive tumor component seems to be an independent negative morphologic prognostic parameter in breast cancer.

Conflict of Interests

The authors declare no conflict of interests.

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Research Article

Basal-Like Phenotype in a Breast Carcinoma Case Series from Sudan: Prevalence and Clinical/Pathological Correlations

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Basal-like breast cancer, an aggressive subtype associated with high grade, poor prognosis, and younger age, is reported frequently in Africa. We analyzed the expression of the basal cytokeratins (CKs) 5/6 and 17 in a case series from Central Sudan and investigated correlations among basal CK status, ER, PgR, and Her-2/neu, and individual/clinicopathological data. Of 113 primary breast cancers 26 (23%), 38 (34%), and 46 (41%) were, respectively, positive for CK5/6, CK17, and combined basal CKs (CK5/6 and/or CK17). Combined basal CK+ status was associated with higher grade ($P < .03$) and inversely correlated with ER ($P < .002$), PgR ($P = .004$) and combined ER and/or PgR ($P < .0002$). Two clusters based on all tested markers were generated by hierarchical cluster analysis and k-mean clustering: I: designated "hormone receptors positive/luminal-like" and II: designated "hormone receptors negative", including both basal-like and Her-2/neu+ tumors. The most important factors for dataset variance were ER status, followed by PgR, CK17, and CK5/6 statuses. Overall basal CKs were expressed in a fraction of cases comparable to that reported for East and West African case series. Lack of associations with age and tumor size may represent a special feature of basal-like breast cancer in Sudan.

1. Introduction

Cytokeratins (CKs) are used as differentiation markers in breast cancer (BC), since their expression is thought to remain stable in carcinogenesis [1]. In breast ducts CK8 and CK18 are expressed in the luminal layer whereas CK5/6, CK14, and CK17 characterize the basal layer [2–4]. Thus BC may be luminal or basal with regard to CK phenotype, with some tumors coexpressing both basal and luminal CKs [2]. This is supported by microarray expression profiling that

classifies BC into five prognostically and clinically relevant molecular subtypes, luminal A, luminal B, basal-like, Her-2/neu, and normal breast-like [5–16]. Accordingly, BC can no longer be viewed as a single biologic and pathologic entity, which implies a need for stratified rather than unified approaches for research, prevention, and treatment [17].

The basal-like subtype overlaps, but is not synonymous, with the triple negative subset, which includes BCs that do not express ER, PgR, and Her-2/neu and tend to occur at a younger age and in patients with pathogenetic *BRCA1* mutations [18–21]. Approximately 85% of the ER–/Her-2/neu– BCs are of basal-like phenotype [9]. Most importantly, although most basal-like BCs do not express ER, PgR, or Her-2/neu, in case series of different origin 14% to 45% of the cases were reported to express at least one of these markers [7, 9, 14].

Basal-like/triple negative BCs initially respond to chemotherapy in the neoadjuvant setting, but their overall prognosis remains poor [14]. Importantly, the tumors with worst prognosis seem to be those expressing basal CKs [5, 7, 8, 22] or epidermal growth factor receptor (EGFR) [9, 23].

Basal-like BCs show common as well as heterogeneous morphologic, genetic, and immunophenotypic features, and, up to date, there is no international consensus regarding their exact definition [5–12, 20]. Basal CKs, which have been shown to be independently associated with poor outcome [7, 9, 24–26], are expressed in most, but not all, BCs classified as basal-like by immunohistochemical (IHC) or gene microarray analysis [3, 7, 20, 27–29]. Furthermore in a subset of BCs basal CKs are coexpressed with other markers, including EGFR, P-cadherin, c-KIT, caveolin 1, and p63, although consideration of such markers does not appear to improve the identification of the cases with poor outcome compared to basal CKs alone [20]. Therefore Rakha et al. [20] suggested to rely on basal CK expression alone to define basal-like BC, remarking that, in spite of shared clinicopathologic and IHC features, basal CK-positive BCs and basal-like BCs are not strictly the same entity [7, 29].

Genetic, ethnic, and racial factors influence BC phenotypes, possibly by determining intrinsic differences in tumor biology [6, 30, 31]. In this regard, it is remarkable that basal-like/triple negative BC appears to be more common in African American women [6, 12, 32] and in BC case series from West and East Africa (range: 22%–34%), where it seems to be also associated with features indicative of poor prognosis [33–36].

In a previous study we found that a BC case series from Khartoum, Central Sudan, was comparable to one from Milan, Northern Italy, in combined hormone receptors status and BC subtypes [37]. Relative to the Italian patients, the Sudanese patients were younger and their tumors were larger, of higher grade and more advanced in stage [37].

We address here the question of the BC subtypes identified by clustering analyses within the Sudanese BC case series. To this end, we re-evaluated, using more sophisticated statistical analyses, the expression of the basal CKs 5/6 (CK5/6) and 17 (CK17) in relation to estrogen/progesterone receptors (ER/PgR), human epidermal growth factor receptor 2 (Her-2/neu), and the available clinicopathological and individual data. We refer in this paper to two designations of BCs with basal subtype: (i) basal CK+, defining BCs that express basal CKs regardless of the expression of other markers [20] and (ii) basal-like, identified by the triple-negative CK-positive profile (ER−/PgR−/Her-2/neu−/basal CK+).

Table 1: Basal cytokeratins in the studied case series.

	Number (%)
CK5/6	
Positive	26 (23)
Negative	87 (77)
CK17	
Positive	38 (34)
Negative	75 (66)
Combined (CK5/6 and/or CK17)	
Positive	46 (41)
Negative	67 (59)

Table 2: Basal breast cancer frequencies in the currently studied case series, according to different designations.

BC basal subtype	Designation	Frequency
Basal CK+	basal CKs+ regardless of the expression of other markers (basal CK+)	46/113 (41%)
Basal-like/triple-negative	triple-negative (ER−/PgR−/Her-2/neu−)	18/113 (15.9%)
Basal-like	triple-negative CK-positive profile (ER−/PgR−/Her-2/neu−/basal CK+)	11/113 (10%)

2. Materials and Methods

2.1. Patients. The study is based on a series of 113 Sudanese cases of primary invasive BC diagnosed between 2004-2005 at the Department of Histopathology & Cytopathology of the Radiation and Isotope Center Khartoum (RICK), Khartoum, Sudan. This series, retrospectively selected to include all consecutively accessioned BCs with available paraffin-embedded material adequate for immunohistochemistry (as determined by immunostaining with control antibodies), was previously used to compare pathological, clinical, and prognostic characteristics of BC in Sudan versus Italy [37]. Exclusion criteria were as follows: (a) in situ carcinomas, (b) sarcomas, and (c) secondary tumors. Overall, the most frequent histotype was invasive ductal carcinoma, which accounted for 101/113 cases (89.4%). Other histotypes were invasive lobular (5/113, 4.4%), mucinous (5/113, 4.4%), medullary (1/113, 0.9%), and Paget's (1/113, 0.9%). Some of the included invasive ductal carcinomas were also associated with other features: (i) inflammatory invasive ductal carcinoma (1/113), (ii) lactating adenoma associated with invasive ductal carcinoma (1/113), (iii) invasive ductal carcinoma with squamoid differentiation (1/113), and (iv) invasive

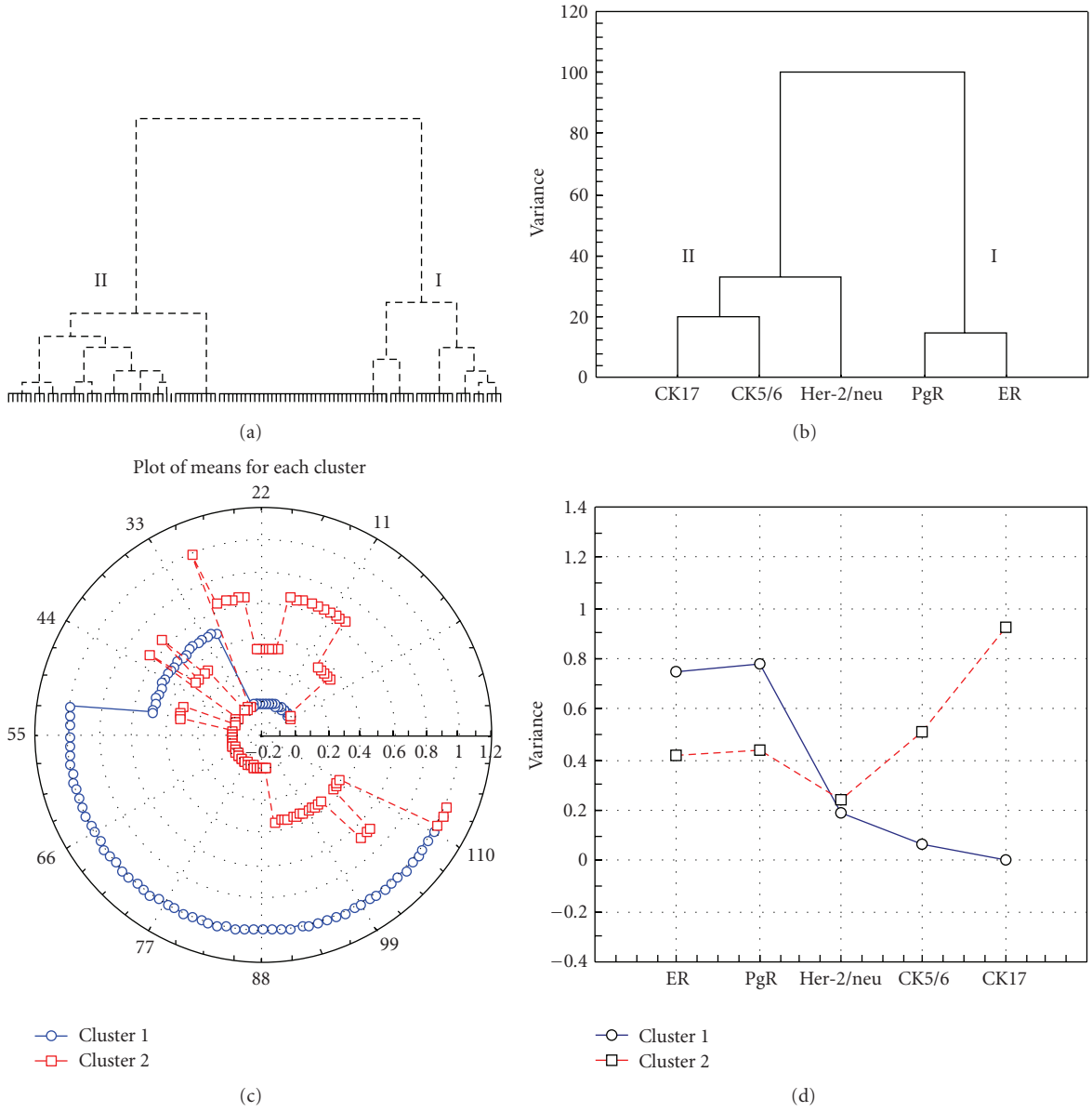

Figure 1: Two clusters generated based on the statuses of basal cytokeratins (CK5/6, CK17), hormone receptors (ER, PgR), and Her-2/neu by hierarchical cluster analysis ((a) & (b)) and k-mean clustering ((c) & (d)). Cases in each cluster are shown in (a) and (c). The factor(s) that contribute to each cluster are shown in (b) and (d).

ductal carcinoma showing features of pleomorphic carcinoma with cartilaginous differentiation (1/113). Histological grading was performed using the Nottingham Combined Histologic Grade (NCHG) system [38]. The breast tumors included in this study were of intermediate grade (grade 2: 35/113; 31%) and high grade (grade 3: 78/113; 69%). The intermediate-grade tumors included all the mucinous carcinomas (5/5, 100%), 3 of the 5 lobular carcinomas (3/5, 60%), and 27/101 (26.7%) of the invasive ductal carcinomas. On the other hand, the high-grade tumors included the unique cases of Paget's and medullary carcinomas and the remaining invasive ductal carcinomas (74/101, 73.3%).

Age and tumor size were recorded only in 73 and 88 of the 113 cases, respectively. Most patients presented with advanced disease and were lost to followup, as it frequently occurs in developing countries [39–41]. Lack of data on lymph node status and follow up precluded correlations with stage and prognosis [37]. According to data from the Sudan Federal Ministry of Health, 78% of the Sudanese BC patients have stage III or IV disease [42, 43].

2.2. Immunohistochemistry. Whole consecutive sections were immunostained for ER (clone 1D5, Dako), PgR (clone PgR 636, Dako), Her-2/neu (polyclonal, Dako), CK5/6 (clone D5/16 B4, Dako), CK17 (clone E3, Dako) and, as quality controls of antigenic preservation, for the CK pool (clones AE1–AE3, Dako) and vimentin (clone V9, Dako). IHC

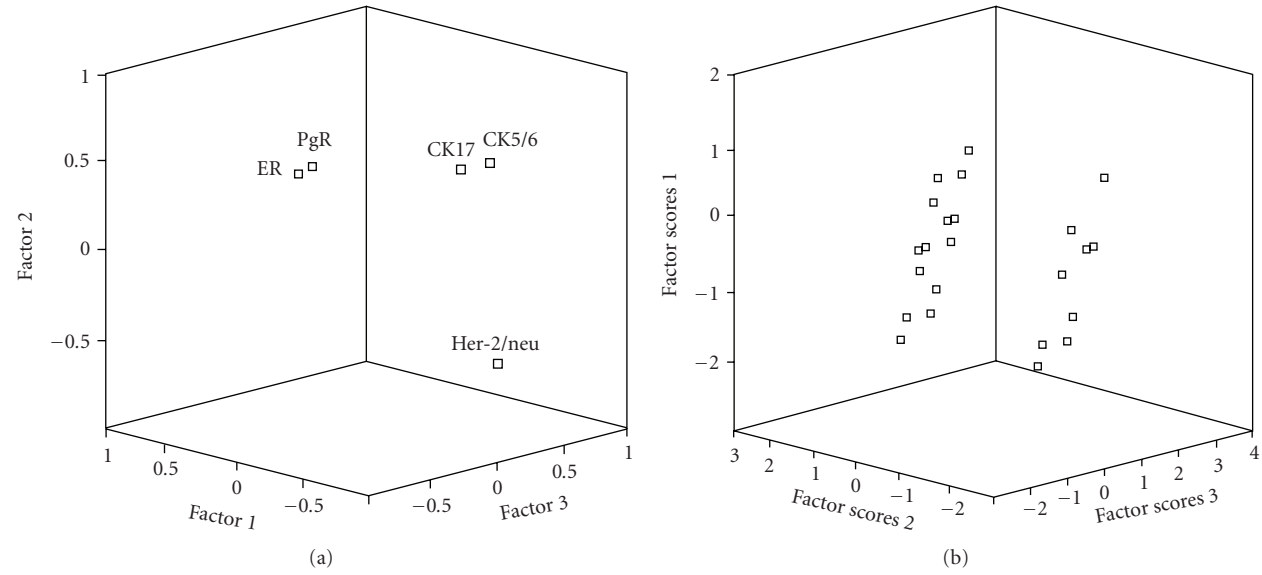

Figure 2: (a) Score components of three factors extracted for the tested dataset variables. Factor analysis showed that three factors explained 80.3% of the dataset variance. The first factor extracted (eigenvalue = 2.1) accounted for the largest proportion of variance (42.3%) and corresponded to hormone receptor status (with loads of ER: 0.80 and PgR: 0.78). The second factor (eigenvalue = 1.2) explained 23.4% of variance and corresponded to basal cytokeratins status (with loads of CK17: 0.55 and CK5/6: 0.54). The third factor (eigenvalue = 0.7, with a load of 0.6 for Her-2/neu status), a factor that explained 14.6% of the variance. (b) Individual factor scores of the three of the five extracted factors. Note that some samples were superimposed. Factor scores were extracted by regression method.

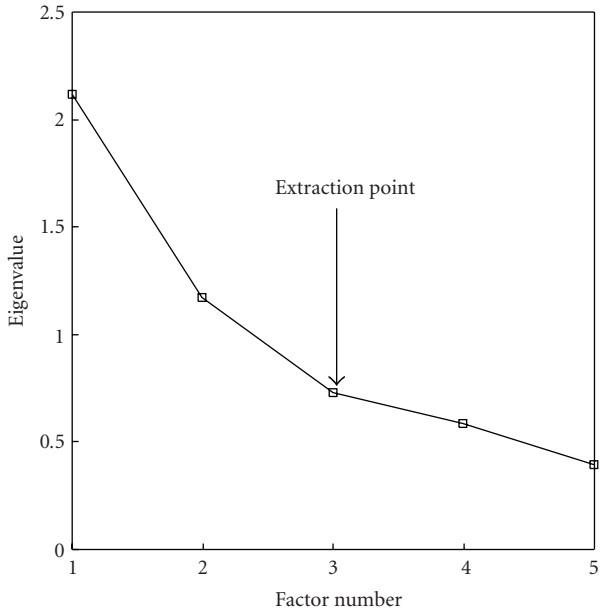

Figure 3: Scree plot of the eigenvalues. The adopted extraction methods were the Kaiser criterion, that is, the sum of squared factor loadings (eigenvalue) >1, and the scree test, that is, the place where the smooth decrease of eigenvalues appears to level off to the right of the plot of the eigenvalues.

results were recorded as percentages of immunostained cells in ≥2000 neoplastic cells. Only nuclear reactivity was taken into account for ER and PR, which were classified as negative, when absent or present in <5% of the neoplastic cells, or positive, when present in ≥5% of the neoplastic cells. Only intense and complete cell membrane immunoreactivity in ≥10% of the cells was taken as evidence of Her-2/neu overexpression (score 3+) [44]. Borderline Her2/neu cases (score 2+) were reassessed by fluorescence in situ hybridization (FISH), as previously described [37]. Basal CKs 5/6 and 17 were regarded as positive when any cytoplasmic and/or cell membrane staining was seen [6, 9, 37].

3. Statistical Analyses

Unsupervised hierarchical cluster analysis (CA) was done for hormone receptors (ER, PgR), Her-2/neu and basal CK (CK5/6 and/or CK17) statuses to determine the natural clustering of the BCs according to the studied IHC markers. CA was performed using squared Euclidean distance measurements to obtain a dissimilarity matrix. Ward's method was then applied to this matrix to build a tree [45]. This method uses analysis of variance to evaluate distances between clusters, minimizing the sum of squares of any two hypothetical clusters that can be formed at each step. CA was done using SPSS statistical package version 15.0 (SPSS Inc., Chicago, IL).

Unsupervised k-mean clustering algorithm, performed with STATISTICA 7.0 (StatSoft, Inc., Tulsa, Ok), was applied to confirm and explore better the generated cluster(s). The k-mean clustering used the Euclidean distance as the similarity metric [46].

Data reduction was done by factor analysis, applying principal components analysis (PCA) to the selected variables (ER, PgR, Her-2/neu, CK5/6, and CK17) to determine

Table 3: Basal cytokeratins status according to tumor grade, tumor size (T), ER, PgR, combined ER/PgR, Her-2/neu, and histology.

	CK5/6+ and/or CK17+, Number (%)		
	Positive	Negative	χ^2
Grade			
g2	9 (20)	26 (39)	4.72 ($P < .03$)
g3	37 (80)	41 (61)	
Tumor size (T)			
$T1$	4 (9)	6 (9)	
$T2$	21 (45.5)	25 (37)	
$T3$	7 (15)	13 (19.5)	0.67 ($P = .88$)
$T4$	5 (11)	7 (10.5)	
NA¶	9 (19.5)	16 (24)	
ER			
ER+	21 (46)	50 (75)	9.8 ($P < .002$)
ER−	25 (54)	17 (25)	
PgR			
PgR+	23 (50)	51 (76)	8.2 ($P = .004$)
PgR−	23 (50)	16 (24)	
Combined ER/PgR			
ER+ and/or PgR+	25 (54)	58 (87)	14.5 ($P < .0002$)
ER−/PgR−	21 (46)	9 (13)	
Her-2/neu			
Her-2/neu+	10 (22)	14 (21)	0.012 ($P = .9$)
Her-2/neu−	36 (78)	53 (79)	
Histology			
IDC*	45 (98)	56 (84%)	
ILC°	—	5 (7%)	
Mucinous	1 (2)	4 (6%)	6.3 ($P = .17$)
Medullary	—	1 (1.5%)	
Paget's disease	—	1 (1.5%)	

¶NA: not available tumor size data in 25 cases, *IDC: infiltrating ductal carcinoma, °ILC: infiltrating lobular carcinoma.

the minimum number of factors, among those considered, that retained most of the dataset variance, and to quantify the significance of the explained variance for each variable in dataset grouping(s). A scoring algorithm, that loaded each individual variable most strongly onto the factor with which it was most correlated, created summary factors. The adopted extraction methods were the Kaiser criterion, that is, the sum of squared factor loadings (eigenvalue) >1 [47] and the scree test, that identifies the cut-off discriminating important from unimportant factors in the plot of the eigenvalues [48]. A default setting of 25 maximum iterations of algorithm steps to obtain convergence was used to extract factors. Factor scores were shown graphically. Statistical analyses were developed by SPSS statistical package version 15.0 (SPSS Inc., Chicago, IL). Factor score loadings were interpreted by rule of thumb in confirmatory factor analysis as follows: ≥0.7: higher factor; <0.7–≥0.6: high factor; <0.6–≥0.4: central factor; <0.4–≥0.25: low factor; <0.25: lower factors [49, 50]. Higher factors build on the rationale that the 0.7 level corresponds to about half of the variance in the indicator being explained by the factor. However, being the 0.7 standard high for real-life data, for exploratory purposes lower levels were used, down to 0.7, with 0.4 for the central factor and 0.25 for other factors [49, 50].

All cut-off values were determined before the statistical procedures. Correlations between different variables were calculated using χ^2 test or t-test. Significance was set at <.05. All P values were two-tailed.

4. Results

4.1. Immunohistochemical Characteristics and Basal Cytokeratin Status. Table 1 summarizes the basal cytokeratin status in the studied case series. Of 113 primary BCs 26 (23%), 38 (34%), 18 (16%), and 46 (41%) were respectively positive for CK5/6, for CK17, for CK5/6 and CK17, and for CK5/6 and/or CK17. The frequency of the basal CK+ subtype (basal CKs+ regardless of other markers) was therefore 46/113 (41%), whereas the basal-like subtype as defined by triple-negative CK+ profile (ER−/PgR−/Her-2/neu−/basal CK+) was 11/113 (10%). Moreover, the frequency of basal-like subtype as synonymous of triple negative, regardless of CK status, was 18/113 (15.9%) (Table 2). Combined positive basal CK status (CK5/6+ and/or CK17+) was associated with higher grade ($P < .03$, Table 3) and was inversely correlated with the expression of ER and PgR (resp., $r = -0.3$, $P < .002$; $r = -0.27$, $P = .004$, Table 3). A highly significant negative correlation emerged when combined hormone receptor status (ER+ and/or PgR+) was considered ($r = -0.36$, $P < .0002$, Table 3).

There was no association between basal CK status and Her-2/neu (Table 3). However, as basal CK+ status, Her-2/neu+ status was inversely correlated with the expression of ER and PgR (resp., $r = -0.27$, $P = .004$; $r = -0.26$, $P = .005$), and with combined ER+ and/or PgR+ status ($r = -0.28$, $P = .003$). Basal CK status was not associated with age at diagnosis (available for 73 cases) and tumor size (available for 88 cases) (Tables 3 and 4); however, although not significant, the mean age of the patients with basal CK+ tumors was lower compared to that of the patients with basal CK− tumors (49.8 ± 15.8 years versus 51.2 ± 14.1 years, Table 4), and the mean tumor size was smaller (4.5 ± 2.7 cm versus 5.4 ± 3.4 cm, Table 4). All the lobular (5/113) and mucinous tumors (5/113) were ER+/PgR+/Her-2/neu− (luminal type) and all were negative for the basal CKs, except one mucinous tumor that was found to be positive for CK5/6. The unique cases of Paget's (1/113) and medullary (1/113) carcinomas were both found to be ER−/PgR−/Her-2/neu+/basal CK−(Her-2/neu subtype).

Therefore, the tumors positive for the basal CKs were invasive duct carcinomas (98%), except a single mucinous carcinoma (Table 3). No association emerged between basal CKs expression and BC histotype (Table 3).

4.2. Cluster Distribution and Factor Analysis. Two major clusters of patients were generated using hierarchical cluster analysis (Figure 1(a)): cluster I with 65/113 (57.5%) patients

Table 4: Basal cytokeratins status according to patient's age at disease diagnosis and to tumor size.

	CK5/6 and/or CK17		
	Positive	Negative	t-test
Age (years)*			
Mean ± SD¶	49.8 ± 15.8	51.2 ± 14.1	($t = 0.57$; $P = .57$; 95% CI −5.6–9.08)
Range	25–80	30–70	
Mean tumor size (cm)#			
Mean ± SD	4.5 ± 2.7	5.4 ± 3.4	($t = 0.58$; $P = .56$; 95% CI −0.85 –1.55)
Range	1–15	1–14	

*The mean age of this series was 51.2 ± 14.3 years (range: 25–80 years), age was missing for 40 cases. ¶SD: standard deviation; #the mean tumor size of this series was 4.7 ± 2.8 cm (range: 1–15 cm), size was missing for 25 cases.

Table 5: Component matrix of the five factors extracted by principal component analysis (PCA).

	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5
ER	0.804	0.228	0.288	0.134	0.448
PgR	0.784	0.252	0.366	−0.015	−0.433
Her-2/neu	−0.391	−0.678	0.612	0.112	0.012
CK5/6	−0.585	0.538	0.351	−0.488	0.080
CK17	−0.598	0.553	0.123	0.564	−0.051

and cluster II with 48/113 (42.5%) patients. Clustering the five tested IHC markers revealed that hormone receptors (ER, PgR) clustered in I whereas the basal CKs (CK 5/6, CK 17, and Her-2/neu clustered in II, each in a separated branch (Figure 1(b)). Hence, cluster I could be designated as "hormone receptors positive/luminal-like," whereas cluster II as "hormone receptors negative," including both the basal-like and the Her-2/neu+ subtypes [5, 22, 26].

Comparable results were obtained through k-mean clustering, with 72/113 (63.7%) patients joining cluster I and 41/113 (36.3%) cluster II (Figure 1(c)). In addition k-mean clustering revealed that hormone receptors (ER, PgR) and basal CKs (CK 5/6, CK 17) played a major role in identifying clusters I and II, respectively (Figure 1(d)). On the other hand, Her-2/neu played quite similar roles in the determination of the two clusters, with slightly higher weight in cluster II (Figure 1(d)).

Factor analysis showed that three factors explained 80.3% of the dataset variance (Figures 2(a) and 2(b)). The first factor (eigenvalue = 2.1) accounted for the largest proportion of variance (42.3%) and corresponded to hormone receptor status (loads: ER: 0.80; PgR: 0.78), while basal CKs (loads: CK17: −0.6: CK5/6, −0.59) and Her-2/neu (load: −0.39) statuses were negatively loaded on this factor. The second factor (eigenvalue = 1.2) explained 23.4% of variance and corresponded to basal CK status (loads: CK17, 0.55; CK5/6, 0.54), while Her-2/neu status (load: −0.68) loaded negatively on this factor. The third factor, corresponding to Her-2/neu status (eigenvalue = 0.7, with a load of 0.6), explained 14.6% of the variance (Figure 2(a)). Individual factor scores of the extracted factors are shown in Figure 2(b). Other two factors needed to be extracted to explain the complete dataset variance, that is, factor 4, corresponding to CK17 status (eigenvalue = 0.6, load: 0.56), that explained 11.7% of the variance, while CK5/6 (load: −0.49) loaded negatively on this factor and factor 5, corresponding to ER status (eigenvalue = 0.4, load: 0.45), that explained 8% of the variance, while PgR (load: −0.43) loaded negatively on this factor. The Scree plot of the eigenvalues is shown in Figure 3. The component matrix of these five factors is shown in Table 5. Of note, these analyses are in support of the proposal of Rakha et al. [20] who suggested to rely on basal CK expression alone (basal CK+ subtype) to define basal-like BC, regardless of the status of the other markers. In fact, our analyses assigned all the BCs that expressed basal CKs, regardless of the other markers, to cluster II. Furthermore, the basal-like subtype (BCs with triple-negative phenotype that express basal CKs: ER−/PgR−/Her-2/meu−/basal CKs+) was also included in cluster II. It is worth mentioning that the adoption of the latter criterion only for the definition of basal BC would miss many cases, as the basal-like subset accounted for only 10% of the cases versus 41% for the basal CKs+ subset.

5. Discussion

The expression of basal CKs is a negative prognostic marker, implying resistance to therapy and poor prognosis, particularly in the context of BCs with triple-negative status [12, 25, 26, 35, 51]. Basal-like BC, which largely overlaps with triple-negative BC, is a well-recognized BC subtype with the above-mentioned clinically-relevant implications [12, 25, 26, 35, 51]. Basal-like/triple-negative BC appears to occur more frequently in African American women and in breast cancer case series from East and West Africa, which could reflect intrinsic differences in tumor biology related to racial/ethnic factors [6, 12, 21, 30, 32].

A better understanding of the impact of basal-like/triple negative BC in BC series from native African women would contribute to the assessment of the influence of race on this particularly relevant BC subtype. It is important to develop BC prevention and treatment policies in African populations, that, with increased life expectancy, are predicted to face marked increases in BC rates [12, 14, 28, 35, 52, 53].

Recent studies found that the basal-like phenotype was frequent in West (Nigeria and Senegal) and East (Uganda) African BC case series (range: 22% to 27%), where it was also associated with features of poor prognosis [33–36]. In

contrast, we [37] and Adebamowo et al. [54] reported lower frequencies of basal-like BC subtype (as defined by triple-negative, basal CK+ phenotype) in Sudanese (10%) and Nigerian BC series (15.8%), which was mainly due to the markedly higher frequency of hormone receptor positivity found in these tumor series (Sudan: ER: 64%; PgR: 67%; ER and/or ER: 75%, Nigeria: ER+: 65.1%; PgR: 54.7%), as compared to the other studies from Africa [33–37, 54].

Consideration of two basal subtypes, that is, basal CK+, defined by expression of basal CKs regardless of other markers [20], and basal-like, defined by the triple-negative CK+ phenotype (ER−/PgR−/Her-2/neu−/basal CK+), may explain these discrepancies. In fact, in our BC series from Central Sudan, the frequency of basal-like BC is 10%, as previously reported [37], but that of basal CK+ BC is 41%. This reflects the presence of an excess of cases that express basal CKs together with ER/PgR and/or Her-2/neu.

In the present Sudanese BC series the frequency of basal CK+ status (41%) appears to be much higher than those reported for Western Caucasian and also for African American BC series (13–20% and 26%, resp.), but results quite comparable to the 34% frequency found in a BC series from Kyadondo County in Uganda and to the 33% frequency reported from West Africa (Nigeria and Senegal) [20, 25, 29, 33, 35, 51]. In the study of Adebamowo et al., basal CKs were not investigated and the basal-like subtype was defined by triple-negative phenotype only (ER−, PR−, and Her-2/neu−) as one category [54]. In this regard it is notable that the Nigerian and the Sudanese case series yield almost the same frequencies of basal-like BCs defined by triple negative phenotype only: 15.8%, that is, 24/152, in the Nigerian series and 15.9%, that is, 18/113, in the Sudanese series [37, 54].

In our Sudanese series, basal CK expression was associated with higher histologic grade and with hormone receptor negative status. This is in agreement with well-established evidence that the expression of basal markers occurs in poorly differentiated hormone receptors-negative BCs, as reported for Caucasian and African American series and also for the Ugandan series [25, 26, 35, 51, 55]. As in other studies, CK17 was more frequently positive than CK5/6 [25].

It is well established that in both African-American and Caucasian BC series the expression of basal CKs is significantly related to younger age at BC onset [26]. In our Sudanese series basal CK status was not associated with age at disease diagnosis, as also reported for the series from Kyadondo County in Uganda [35]. However, although not significant, the mean age and the mean tumor size were lower in the basal CK+ group than in the basal CK− one. The lack of significance for the difference in age may be due to the fact that the patients were mostly young, reflecting the young age at disease diagnosis typical of the institutional BC series from the Sudan [37, 56–58].

Indeed, the higher frequency of basal-like phenotype in African case series could be partially explained by the younger age of the patients [33–36]. However, socioeconomic, genetic, ethnic, and lifestyle/reproductive factors are also likely to be involved [30, 37]. In particular, emerging data reported that certain reproductive factors (i.e., extended breast-feeding/lactation, high parity, and early menarche) may have a greater impact on risk of certain molecular BC subtypes compared to others [59, 60]. Furthermore, other confounding factors, like antigen degradation of archival formalin fixed, paraffin-embedded tissue blocks, should also be considered for the reportedly high frequency of hormone receptor negativity, with subsequently higher frequencies of both basal-like BC identified by the triple-negative CK+ profile (ER−/PgR−/Her-2/neu−/basal CK+) and unclassified triple-negative types [33, 36, 37, 54, 61].

The lack of association between basal CK+ status and larger tumor size is quite unexpected [51]. This unusual finding might reflect the fact that large size at presentation, due to late disease diagnosis, is one of the main features of BC in Sudanese patients, when compared to BC in patients from Europe and North America [9, 37, 62, 63]. Due to longer survival, this could result in a relative enrichment of less aggressive subtypes among the BCs of larger size [37, 64], a hypothesis that requires to be further investigated in larger and prognostically well-characterized BC series from Sudan.

Except one mucinous carcinoma, all the basal CK+ tumors were invasive duct carcinomas, consistent with the literature data [51]. The fact that all the invasive lobular tumors were basal CK− could be relevant but could also reflect a bias due to the relatively low frequency of this histotype in the study series and needs further evaluation on a larger number of cases.

In concordance with the gene expression-based IHC subtypes defined in Western BC case series [5, 22, 26], clustering based on the five tested IHC markers outlined a hormone receptors-positive/luminal-like cluster and a hormone receptors-negative cluster with basal CKs (CK5/6, CK17) and Her-2/neu. As expected, factor analysis showed that hormone receptor status was the factor that most influenced dataset variance among the other tested factors, being negatively affected by both basal CK and Her-2/neu statuses. Basal CK status was in second position, with Her-2/neu status loaded negatively on this factor, although this was not supported by a direct negative correlation. Her-2/neu status was in the third place. The other two extracted factors (factor 4: CK17 status, and factor 5: ER status) had minimum effects as extracted factors on the dataset variance. Collectively, this demonstrates that the most important factors in the dataset were ER status, followed by PgR, CK17, and CK5/6 statuses.

Her-2/neu status played a complex role in the dataset variance, as it negatively affected both hormone receptor status (which was consistent with statistical correlations) and basal CK status (as demonstrated only by factor analysis). As previously reported, the basal-like phenotype and the Her-2/neu expression are inversely correlated [9, 14, 65, 66], and it is likely that the nonbasal-like tumors include a high prevalence of Her-2/neu amplified tumors [65]. In this regard, it should be considered that the effects of Her-2/neu on the determination of the two clusters were quite similar, being only slightly in favour of cluster II (Figure 1(d)). Interestingly, Harris et al. reported that the expression of basal markers was strongly associated with Her-2/neu+ BCs not responding to preoperative therapy based on

trastuzumab plus vinorelbine [53]. This underlines the need to better verify the BC subsets in which basal CKs, Her-2/neu and hormone receptors could interact, in African and non-African case series.

6. Conclusion

In the presently studied BC series from Central Sudan the frequency of the tumors expressing basal CKs was much higher than the frequencies reported for Caucasian and African-American BC series, but it was comparable to that found in BC series from East and West Africa [20, 25, 29, 33, 35, 51]. This suggests that the impact of the tumors expressing basal CKs could be higher in sub-Saharan African patients, a possibility that needs to be confirmed by additional studies in different African populations. In Sudan a higher impact of the tumors expressing basal CKs could be ascribed to a variety of factors, including racial/genetic factors, environmental and reproductive factors, population structure, and sampling/referral bias. However, while an early age of onset is one of the clinical characteristics associated with BC expressing basal CKs, in our case series basal CK-positive status was associated with higher grade and hormone receptor-negative status, but not with age at disease diagnosis and tumor size. This quite unexpected lack of association might reflect a selective effect of late disease diagnosis. The most important factors for clusterization in distinct BC subsets were ER status, followed by PgR, CK17, and CK5/6 statuses. As in West Africa, the identified clusters were in concordance with the gene expression-based immunohistochemical subtypes defined in Western BC case series [5, 22, 26, 33], despite the difference in patient population. However, the overall frequency of basal-like subtype (ER−/PgR−/Her-2/neu−/basal CK+) was low (10%, in Sudanese; 15.8%, in Nigerian), which was mainly due to the reported markedly higher frequency of hormone receptor positivity (ER: 64%; PgR: 67%; ER and/or ER: 75% in Sudanese and ER+: 65.1%; PgR: 54.7% in Nigerian) as compared to the other studies from Africa [33–37, 54].

Conflict of Interests

The authors have no conflict of interests to declare.

Acknowledgments

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Research Article

ER81 Expression in Breast Cancers and Hyperplasia

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ER81 is a transcription factor that may contribute to breast cancer; however, little known about the role of ER81 in breast carcinogenesis. To investigate the role of ER81 in breast carcinogenesis, we examined ER81 expression in IDC, DCIS, ADH, HUT, and normal breast tissues by immunohistochemical staining. We found that ER81 overexpression was detected in 25.7% (9/35) of HUT, 41.2% (7/17) of ADH, 54.5% (12/22) of DCIS, and 63.0% (51/81) of IDC. In 20 of breast cancer tissues combined with DCIS, ADH, and HUT, ER81 expression was found in 14/20 (70%) IDC. In these 14 cases all cases were ER81 positive expression in DCIS, 13 of 14 cases were positively expressed of ER81 in ADH and 8 of 14 were positive for ER81 in HUT components. A statistical significance was found between NBT and HUT ($P < .05$) and HUT and ADH ($P < .05$). Clinical-pathological features analysis of breast cancer revealed that ER81 expression was significantly associated with Her2 amplification and was negatively associated with ER and PR expression. Our results demonstrated that ER81 overexpression was present in the early stage of breast development that suggested that ER81 overexpression may play an important role in breast carcinogenesis.

1. Introduction

Breast carcinogenesis is thought to undergo a transition from normal epithelium to invasive carcinoma (IDC) via hyperplasia of usual type (HUT), atypical ductal hyperplasia (ADH), and carcinoma in situ (DCIS) [1]. Over 14% of breast cancer diagnosed in the United States annually are DCIS, and approximately 50% of untreated DCIS will develop into an IDC within 24 years after the original biopsy [2]. However, it is unclear how IDC develop from these lesions.

ER81 (ETS-related 81), also called ETS variant 1 (ETV1), is a transcription factor that is a member of the ETS family of DNA-binding proteins [3–5]. Its association with cancer was first noted in Ewing tumors, in which the EWS gene can be translocated onto the ER81 gene and the resultant EWS-ER81 fusion protein exerts oncogenic properties [6]. From then on, many findings suggest that dysregulation of ER81 target genes in disparate tumors like Ewing sarcomas and prostate carcinomas are causally involved in tumorigenesis [7]. Of note, ER81 transcriptional activity is dramatically enhanced upon Her2/Neu (a receptor tyrosine kinase and proto-oncoprotein especially associated with breast cancer) overexpression [8, 9]. Moreover, ER81 mRNA levels are increased in murine cell lines and tumors overexpressing Her2/Neu and also in many human breast cancer cell lines, which suggests that ER81 may contribute to breast tumorigenesis [10–12].

To gain more insight into ER81's role in breast tumorigenesis, we attempted to examine ER81 expression in invasive breast cancers, ductal carcinoma in situ, atypical ductal hyperplasia, and hyperplasia without atypia and normal breast tissues in this study.

2. Materials and Methods

2.1. Materials. Formalin-fixed, paraffin-embedded tissue specimens including 103 of primary breast cancers including 81 invasive ductal carcinomas (IDC) and 22 ductal carcinomas in situ (DCIS), 52 of breast hyperplasia including

17 atypical ductal hyperplasia (ADH) and 35 hyperplasia of usual type (HUT), and 62 of normal breast tissues (NBT) were collected from Department of Pathology, Kunming General Hospital and The First People's Hospital of Yunnan Province between June 2006 and October 2009. Furthermore, 20 paraffin-embedded tissue blocks of breast invasive ductal carcinomas combined with ductal carcinomas in situ, atypical Hyperplasia, hyperplasia of usual type, and normal breast tissues were got from the two hospitals.

2.2. Immunohistochemical Staining. Immunohistochemistry was employed to detect the expression of ER81 for all breast tissues and the expression of ER, PR for all the 81 cases of invasive ductal carcinomas tissues. EnVision Systems was adopted for the staining. Briefly, 4 μm sections were taken from formalin-fixed, paraffin-embedded tissue blocks. The deparaffinized sections were pretreated with heat-induced epitope retrieval and then treated with 30 mL/L hydrogen peroxidase in methanol for 30 min to block endogenous peroxidase activity. The sections were further blocked with 10 mL/L normal goat serum for 30 min, followed by incubation with primary antibody (mouse antihuman monoclonal antibody ER81, SANTA CRUZ BIOTECHNOLOGY, INC; mouse antihuman monoclonal antibody ER and PR, Maixin-Bio, Fuzhou, China) at 4°C overnight. The sections were then washed in 0.01 mol/L phosphate buffer solutions (PBS, pH 7.2) and sequentially incubated with Envision (Envision kit, DakoCytomation, Inc, Carpinteria, California, USA) for 30 min. The reaction product was visualized by diaminobenzidine tetrahydrochloride (DAB). All slides were counterstained with hematoxylin, dehydrated, and mounted. PBS substituting for the primary antibody was used as the negative control.

2.3. Assessment of Immunohistochemical Staining. Specific staining was evaluated independently by two investigators. We used a semiquantitative manner to assess the ER81 staining, yielding an immunoreactive score (IRS) ranging from 0 to 9. IRS was calculated by multiplying the number of positive cytoplasmic staining of cells (0 = none, 1 =< 10%, 2 = 10–50%, 3 => 50% positive tumor cells) by the staining intensity (1 = weak, 2 = moderate, 3 = strong). Then we considered IRS 0 score as ER81 expression "−", IRS 1-2 score as ER81 expression "+", IRS 3–5 score as ER81 expression "++", IRS 6–9 score as ER81 expression "+++". Positive reaction in a normal epithelium yielded a maximum IRS of 2. Therefore breast hyperplastic cells and breast cancer cells in other groups were considered ER81 positive with IRS $\geq$ 3 as suggested by Going et al. [13]. The ER and PR positive staining should be localized to the nucleus. Specimens in which more than 10% of cells showed positive immunoreactivity were considered to be immunoreactive.

2.4. Fluorescence In Situ Hybridization (FISH). A HER2/neu probe kit (China Medical Technologies, Inc, Beijing, China) was used for FISH analysis for all the 81 cases of invasive ductal carcinomas tissues. Tissue sections were baked overnight at 56°C, dewaxed in xylene, dehydrated and air-dried. The slides were then pretreated with sodium bisulfite at 50°C for 30 min and digested with protease K for 15 min at 37°C and finally hybridized overnight at 42°C with the probes (GLP HER2/CSP17 DNA probe, China Medical Technologies, Inc, Beijing, China) after DNA denaturation at 73°C. Slides were washed with posthybridization buffer at 73°C, counterstained with 4, 6-diamidino-2-phenylindole (DAPI) and mounted and stored in the dark prior to signal enumeration. For FISH analysis, slides were examined with fluorescence microscope. Areas of optimal tissue digestion and no overlapping nuclei were then selected in each core for counting. 30 cells were counted for each case. We considered cases with a FISH ratio (Her2 gene signals to chromosome 17 signals) of $\geq$2.2 as Her2 amplified.

Table 1: ER81 expression in breast cancers and hyperplasia (n, %).

	−	+	++	+++	Total
NBT	45 (72.6)	17 (27.4)	0 (0.0)	0 (0.0)	62
HUT	6 (17.1)	20 (57.1)	9 (25.8)	0 (0.0)	35
ADH	2 (11.8)	8 (47.1)	6 (35.3)	1 (5.9)	17
DCIS	2 (9.1)	8 (36.4)	8 (36.4)	4 (18.2)	22
IDC	2 (2.5)	28 (34.6)	32 (39.5)	19 (23.4)	81

2.5. Statistical Analysis. The statistical analysis was performed using the SPSS software package, version 11.0. The differences were analyzed by Kruskal-Wallis (K-W or H) test and Pearson chi-squared distribution (χ^2) test. A value of $P < .05$ was considered statistically significant.

3. Results

Formalin-fixed tissue sections from a spectrum of mammary lesions were analyzed for ER81 expression. As shown in Table 1 and Figures 1, 2, and 3, 72.6% normal breast specimens were completely negative for reactivity to ER81, and the rest of the cases reacted very slightly yielding a maximum IRS of 2. Hyperplastic lesions without atypical demonstrated slightly higher levels of ER81 expression than did in nonhyperplasia ($P < .01$) with an average IRS of 1.77 for ER81. In 9 of 35 specimens, hyperplastic epithelium demonstrated IRS = 3, whereas all of the remaining cases were IRS $\leq$ 2. Hyperplastic lesions with atypical generally demonstrated slightly higher levels of ER81 expression than did in nonatypical hyperplastic epithelium ($P < .01$) and nonhyperplasia epithelium ($P < .01$) with an average IRS of 2.53 for ER81. 6 of 17 atypical hyperplasia cases were ER81 expression "++" and 1 case was ER81 expression "+++" with IRS = 6. Ductal carcinoma in situ tissues generally demonstrated slightly higher ER81 expression than did in atypical hyperplasia with an average IRS of 3.18 for ER81, but the difference is not statistically significant ($P > .05$). 4 of 22 (18.2%) ductal carcinoma in situ tissues was ER81 expression "+++" and 8 of 22 (36.4%) cases were ER81 expression "++". Invasive ductal carcinomas demonstrated enhanced levels of ER81 expression. The expression of ER81 in invasive ductal carcinomas was statistically different from that in

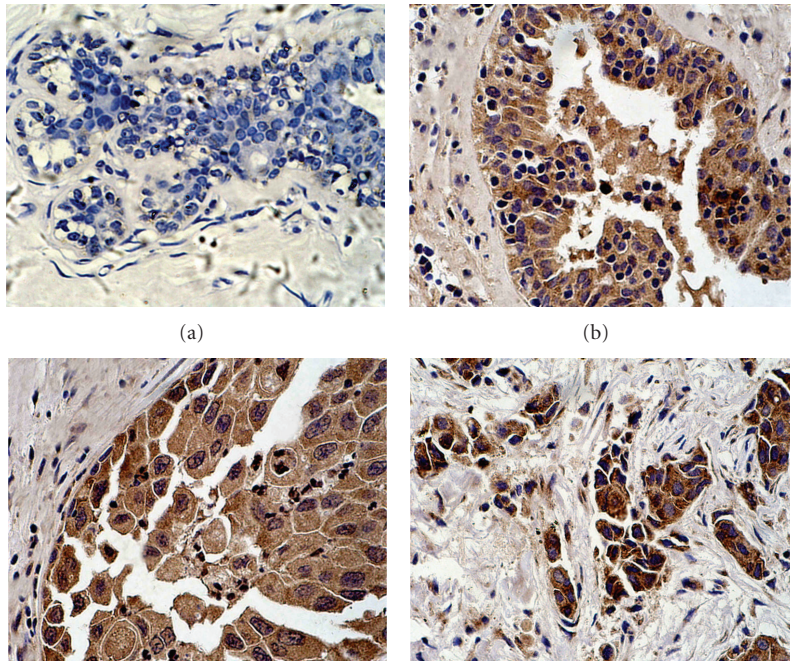

Figure 1: ER81 expression in breast cancers and hyperplasia. (a) Negative expression of ER81 in hyperplasia of usual type (×400). (b) Positive expression of ER81 in atypical ductal hyperplasia (×400). (c) Positive expression of ER81 in ductal carcinoma in situ (×400). (d) Positive expression of ER81 in invasive ductal carcinoma (×400).

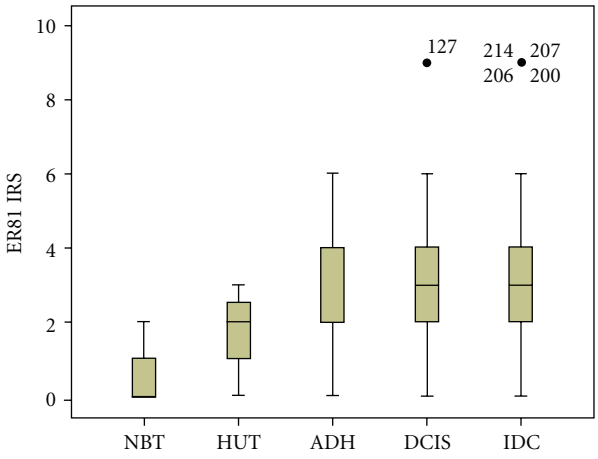

Figure 2: Comparison of ER81 expression in breast cancers and hyperplasia. The box plot markings represent median, 25–75th percentile, and the range of all values.

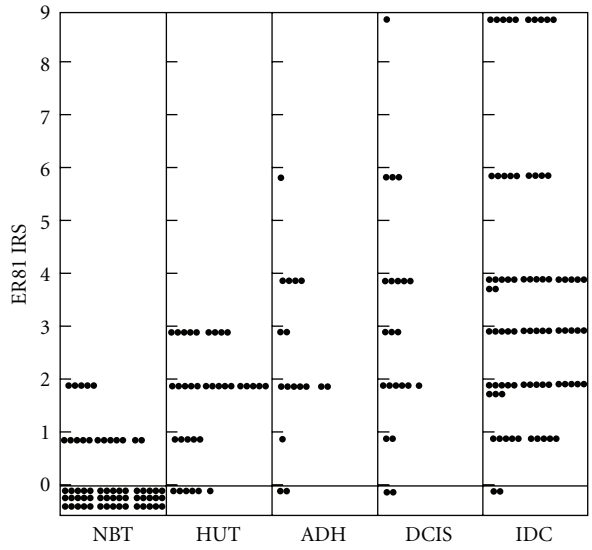

Figure 3: The distribution of ER81 expression in breast cancers and hyperplasia. Each dot represents ER81 IRS of one case.

ductal carcinomas in situ, atypical ductal hyperplasia tissues, hyperplasia cases of usual type, and normal breast tissues ($P < .01$). The average IRS of invasive ductal carcinomas for ER81 was 3.74, and 19 of 81 cases (23.4%) showed ER81 expression "+++". The IRS of carcinoma cells varied from 0 to 9 with 51 of 81 cases (62.9%) demonstrating IRS ≥ 3 for ER81 expression.

The above data provided lines of evidence that ER81 overexpression happened in the early stage of breast cancer development. In order to confirm the association between ER81 expression and breast tumorigenesis, we employed 20 IDC tissues combined with DCIS, ADH, HUT, and NBT to examine ER81 expression. We considered a threshold IRS set at ≥3 as ER81 positive expression. In this group, ER81 expression was found in 14/20 (70%) IDC. In these 14 cases, ER81 expression was found in 14/14 of adjacent DCIS,

Table 2: ER81 expression in breast cancers combined with DCIS and benign breast hyperplasia (n, %).

	−	+	++	+++	Total
NBT	5 (25.0)	15 (75.0)	0 (0.0)	0 (0.0)	20
HUT	4 (20.0)	8 (40.0)	8 (40.0)	0 (0.0)	20
ADH	3 (15.0)	2 (10.0)	11 (55.0)	4 (20.0)	20
DCIS	2 (10.0)	2 (10.0)	10 (50.0)	6 (30.0)	20
IDC	1 (5.0)	5 (25.0)	8 (40.0)	6 (30.0)	20

13/14 adjacent ADH, and 8/14 adjacent HUT components. Adjacent normal breast component demonstrated ER81 negative expression. By K-W analysis for the expression level of ER81, we found a statistical significance between NBT and HUT ($P < .05$) and HUT and ADH ($P < .05$), but no statistical significance was found between ADH and DCIS ($P > .05$) or DCIS and IDC ($P > .05$) (Table 2 and Figures 4, 5).

The relationship between ER81 expression and clinical-pathological features such as ER, PR, and Her2 in breast cancer is listed in Table 3. The results revealed that ER81 expression was significantly associated with Her2 amplification and was negatively associated with ER and PR expression. No correlation was found between ER81 expression and patient ages, menopause status, tumor sizes, nodal status, and histological stage (Table 3).

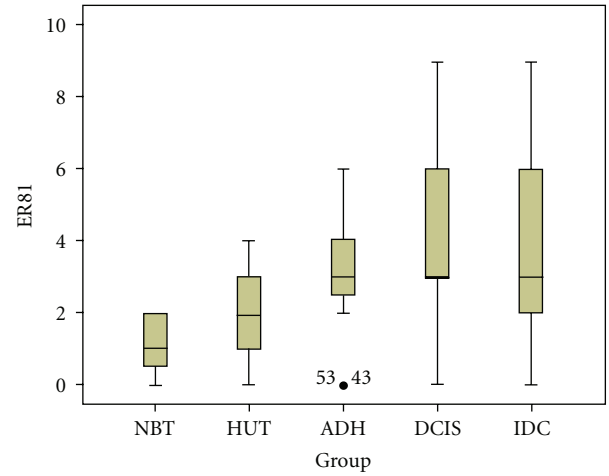

Figure 4: Comparison of ER81 expression in breast cancers combined with DCIS and benign breast hyperplasia. The box plot markings represent median, 25–75th percentile, and the range of all values.

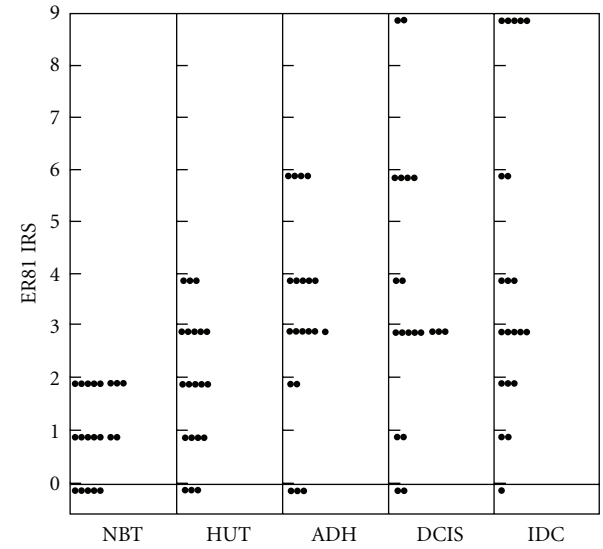

Figure 5: The distribution of ER81 expression in breast cancers combined with DCIS and benign breast hyperplasia. Each dot represents ER81 IRS of one case.

4. Discussions

Increasing lines of evidence suggest that breast cancer develop through a multistep model of carcinogenesis, that is, from normal breast epithelia to hyperplasia without atypia, hyperplasia with atypia, ductal carcinoma in situ, to invasive carcinoma [1, 14, 15]. In experiments carried out by DeOme et al. [16], when hyperplastic breast alveolar nodules (HAN), the breast epithelial cells infected by murine mammary tumor virus, were transplanted to cleared mammary fat pads, half of them developed into carcinomas by 13–21 weeks, which happened more frequently than normal breast tissues. In breast biopsies harbouring malignancy, infiltrating carcinoma is often found side-by-side with *in situ* carcinoma and/or benign proliferations. These lesions occasionally show morphological transition and continuity with the invasive carcinoma. Karpas et al. [17] evaluated 645 breast biopsies (226 malignant and 419 benign) and found atypical hyperplasia in 62% of malignant biopsies, but only in 4% of benign biopsies. Similarly, Kern and Brooks [18] found a greater incidence of atypical ductal hyperplasia (ADH) in cancer-bearing breasts. Foote and Stewart [19] found that papillary hyperplasia with atypia occurred five times more frequently in the cancerous breasts. In a similar study, Ryan and Coady [20] found that hyperplasia was four times more common in the cancerous breasts. These histopathological studies data provide convincing evidence that some forms of proliferative lesions are often found in association with invasive cancer and that ADH provides a significantly increased relative risk of subsequent invasive carcinoma. However, little is known about the molecular genetic mechanisms involved in the transformation from hyperplasia to cancer, which is important for the early diagnosis and molecularly targeted therapy of breast cancers [21].

ER81 is a downstream gene of *Her2*/Neu, a receptor tyrosine kinase and proto-oncoprotein. And *Her2* is especially associated with breast cancer. Of note, ER81 transcriptional activity is dramatically enhanced upon Her2/Neu overexpression [8, 9]. On the other hand, ER81 can target Her2 and upregulate Her2 expression in breast tumors, suggesting the existence of a feed-forward loop in the upregulation of

Table 3: Comparison between ER81 expression and clinical data in breast cancers.

	n	ER81 negative (%)	ER81 positive (n, %)*	P (χ^2)
Age, years				
≤50	48	22 (45.8)	26 (54.2)	.814
>50	33	16 (48.5)	17 (51.5)	
Menopause				
Before	46	22 (47.8)	24 (52.2)	.483
After	35	14 (40.0)	21 (60.0)	
Tumor size, cm				
≤2	15	7 (46.7)	8 (53.3)	.138
2–5	55	28 (50.9)	27 (49.1)	
>5	11	2 (18.2)	9 (81.8)	
Nodal status				
Negative	43	18 (41.9)	25 (58.1)	.463
Positive	38	19 (50.0)	19 (50.0)	
Histological stage				
I	10	6 (60.0)	4 (40.0)	.246
II	54	14 (25.9)	30 (74.1)	
III	17	6 (35.3)	11 (64.7)	
ER				
Negative	25	1 (4.0)	24 (96.0)	.001
Positive	56	22 (39.3)	34 (60.7)	
PR				
Negative	28	3 (10.7)	25 (89.3)	.010
Positive	53	20 (37.7)	33 (62.3)	
HER2 amplification				
Negative	53	19 (35.8)	34 (64.2)	.041
Positive	28	4 (14.3)	24 (85.7)	

* Breast cancer tissues with IRS ≥ 3 were considered as ER81 positive expression.

HER2/Neu [22]. Moreover, ER81 mRNA levels are increased in murine cell lines and tumors overexpressing Her2/Neu and also in many human breast cancer cell lines, suggesting that ER81 may contribute to breast tumorigenesis [10–12]. Shin et al. found that ER81 downregulation suppresses proliferation of *Her2*-positive MDA-MB-231 breast cancer cells in vitro and tumor formation in vivo, proving for the first time the existence of a critical role of ER81 in breast cancer cell physiology [5]. Although transgenic mice overexpressing ER81 in the breast do not develop mammary tumors, ER81 overexpression may prime breast cells to become malignant, for instance upon additional overexpression of Her2/Neu [5].

In this study, we investigated the role of ER81 in breast carcinogenesis by two steps: (1) examining ER81 overexpression in IDC, DCIS, ADH, HUT, and normal breast tissues which represents different stage of breast cancer development. As a result, weak staining was observed in normal breast tissues yielding a maximum IRS of 2. If cells in other groups were considered ER81 positive with IRS ≥ 3, ER81 overexpression was detected in 25.7% (9/35) of HUT, 41.2% (7/17) of ADH, 54.5% (12/22) of pure DCIS, and 63.0% (51/81) of IDC. Although there was ER81 expression in HUT, all ER81 positive tissues were moderate staining. The expression level of ER81 was increased with the progression of the lesion. It is implied that ER81 overexpression are present in the early stage of breast development. (2) Examining ER81 overexpression in breast cancer and the adjacent hyperplasic components (each component represents one stage of breast cancer development) in a single tumor. In this group, ER81 expression was found in 70% (14/20) IDC. In these 14 cases all cases were ER81 positive expression in DCIS, 13 of 14, cases were positively expressed of ER81 in ADH, and 8 of 14 were positive for ER81 in HUT components. A statistical significance was found between NBT and HUT ($P < .05$) and HUT and ADH ($P < .05$), but no statistical significance was found between ADH and DCIS ($P > .05$) or DCIS and IDC ($P > .05$) confirming that ER81 may involve in breast carcinogenesis.

In addition, we analyzed the relationship between ER81 expression and clinical-pathological features of breast cancer including Her2 amplification and ER, PR expression. The results revealed that ER81 expression was significantly associated with Her2 amplification and was negatively associated with ER and PR expression. No correlation was found between ER81 expression and patient ages, menopause status, tumor sizes, nodal status, and histological stage. In Her2 positive amplification group, the number of ER81 positive expressed cases was more than that in Her2 negative amplification group. As we know, overexpression of ER81 in itself does not lead to breast tumor formation [23], possibly because ER81 requires stimulation in order to become transcriptionally competent and the activation of ER81 is inducible by the *Her2* → *Ras* → *Raf* → *MAP* kinase signaling pathway [9, 24, 25]. These results suggest that *Her2* and ER81 synergize breast carcinogenesis.

Conclusively, ER81 overexpression was present in the early stage of breast development. Together with previous study results, it is suggested that ER81 may play an important role in breast carcinogenesis.

Abbreviations

IDC: invasive ductal carcinoma
DCIS: ductal carcinoma in situ
ADH: atypical ductal hyperplasia
HUT: hyperplasia of usual type
NBT: normal breast tissue
IRS: immunoreactive score
ER: estrogen receptor
PR: progesterone receptor.

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Research Article

Novel Molecular Markers of Malignancy in Histologically Normal and Benign Breast

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To detect the molecular changes of malignancy in histologically normal breast (HNB) tissues, we recently developed a novel 117-gene-malignancy-signature. Here we report validation of our leading malignancy-risk-genes, topoisomerase-2-alpha (TOP2A), minichromosome-maintenance-protein-2 (MCM2) and "budding-uninhibited-by-benzimidazoles-1-homolog-beta" (BUB1B) at the protein level. Using our 117-gene malignancy-signature, we classified 18 fresh-frozen HNB tissues from 18 adult female breast cancer patients into HNB-tissues with low-grade (HNB-LGMA; $N = 9$) and high-grade molecular abnormality (HNB-HGMA; $N = 9$). Archival sections of additional HNB tissues from these patients, and invasive ductal carcinoma (IDC) tissues from six other patients were immunostained for these biomarkers. TOP2A/MCM2 expression was assessed as staining index (%) and BUB1B expression as H-scores (0–300). Increasing TOP2A, MCM2, and BUB1B protein expression from HNB-LGMA to HNB-HGMA tissues to IDCs validated our microarray-based molecular classification of HNB tissues by immunohistochemistry. We also demonstrated an increasing expression of TOP2A protein on an independent test set of HNB/benign/reductionmammoplasties, atypical-ductal-hyperplasia with and without synchronous breast cancer, DCIS and IDC tissues using a custom tissue microarray (TMA). In conclusion, TOP2A, MCM2, and BUB1B proteins are potential molecular biomarkers of malignancy in histologically normal and benign breast tissues. Larger-scale clinical validation studies are needed to further evaluate the clinical utility of these molecular biomarkers.

1. Introduction

Despite recent advances in biomarker discovery, no clinically proven biomarkers of increased breast cancer risk have been identified and validated in histologically normal breast. However, there is increasing evidence in the current literature for the presence of specific genetic abnormalities in histologically normal breast tissue in patients with and without breast cancer [1–10]. Such genetic abnormalities are often common to the tumor and their matched histologically normal breast tissues, suggesting their association with subsequent development of breast cancer in those patients. Whether such

molecular abnormalities are the cause or the effect of the development of breast cancer is largely unknown. Also the degree of expression and microanatomical distribution of these molecular abnormalities in histologically normal/benign breast tissues is still poorly defined.

In order to elucidate the molecular changes of malignancy in HNB tissues, we used the Affymetrix platform to profile a large prospective series of fresh-frozen HNB tissues and invasive ductal breast carcinomas (IDCs). Based on the differential expression of a number of IDC-specific genes in HNB tissues, we developed a novel 117-gene malignancy signature for molecular classification of HNB tissues into two subsets that we designated as those with high-grade and low-grade molecular abnormalities. We subsequently validated our microarray data on HNB tissues using real-time PCR (qPCR) [11] and demonstrated additional utility of our malignancy signature by cross-validation analyses on publically available breast data sets.

Many of the genes in our 117-gene signature were "proliferation genes." Some of these "proliferation genes" (TOP2A, MCM2, and BUB1B) are also important targets for breast cancer therapy. Here we report cross-platform validation of these 3 genes as our leading candidate malignancy genes at the protein level. We used immunohistochemistry on a new set of archival sections of HNB tissues from 18 breast cancer/DCIS/prophylactic mastectomy patients whose breast tissues (cancer and normal) were used to derive our 117-gene malignancy signature in the original microarray experiment. Since the cells lining the terminal duct lobular units (TDLUs) are thought to be the precursor cells of breast cancer [12], in this cross-platform (microarray to IHC) validation analysis we focused primarily on the immunohistochemical expression of TOP2A, MCM2, and BUB1B in the histologically normal TDLUs, although we believe that these proteins may also be useful in the molecular stratification of benign breast disease and premalignant breast lesions such as atypical ductal hyperplasia (ADH).

2. Materials and Methods

2.1. Patients and Specimens. This study was carried out under approval by the Institutional Review Board (IRB) at the University of South Florida, Tampa, FL. It included 24 adult female patients who underwent mastectomy for their breast cancers at Moffitt Cancer Center (MCC), Tampa, FL between 2002 and 2005. Eighteen of these patients had fresh-frozen histologically normal breast (HNB) tissues previously analyzed using Affymetrix Plus 2.0 Gene chip to develop a 117 gene signature to be used for molecular classification of histologically normal breast tissues. Based on the expression levels of 117-genes in our malignancy signature (Figure 1), these 18 specimens were classified as HNB tissues with high-grade and low-grade molecular abnormalities (HNB-HGMA; N=9 and HNB-LGMA; N=9). Mean ages for patients with HNB-HGMA and HNB-LGMA were 50 and 55 years, respectively. Pertinent clinicopathologic data, based on information available from MCC and Contributing Institutions' Surgical Pathology reports, electronic patient records, MCC Cancer Registry, and retrospective review of all available H&E slides from MCC Pathology Archives and outside institutions, is summarized in Table 1.

All available formalin fixed, paraffin-embedded (FFPE) sections from the mastectomies of the study patients (N = 18) were reviewed by an experienced breast pathologist (AN) to select HNB tissue blocks for immunohistochemical validation of 3 of our leading malignancy-risk genes (TOP2A, BUB1B, and MCM2). The selection of FFPE block representative of each HNB tissue was based on the presence of maximum number of histologically normal terminal duct lobule units (TDLUs) on a single H&E. stained section among all of the archival sections reviewed from that patient. Archival tumor sections from 6 other adult female patients (mean patient age: 69 years) with IDCs (Cases 1–6; Table 1) were selected as positive tissue controls to validate the immunohistochemical expression of TOP2A, MCM2, and BUB1B protein on archival sections of HNB tissues. Table 2 compares ages for the 3 patient groups in this analysis.

2.2. TOP2A, MCM2, and BUB1B Protein Immunohistochemistry. Five-micron thick serial FFPE sections from each selected IDC (N = 6), HNB-HGMA (N = 9), and HNB-LGMA (N = 9) tissue block were stained with H&E, and for TOP2A, MCM2, and BUB1B protein proteins, using immunohistochemical (IHC) protocols optimized in the Tissue Core Laboratory at our institute (AN). The IHC staining was carried out using a Ventana Discovery XT automated system (Ventana Medical Systems, Tucson, AZ, USA) as per manufacturer's protocol with proprietary reagents. Briefly, slides were deparaffinized on the automated system with EZ Prep solution (Ventana). Enzymatic retrieval was used with Protease 1 solution (Ventana).

The mouse monoclonal antibody that reacts with human TOP2A protein (#MS-1819-SO, Neomarkers) was used at a 1 : 50 concentration in Dako antibody diluent and incubated for 60 min. The mouse monoclonal antibody that reacts with human MCM2 protein (#MS1726PO, Neomarkers) was used at a 1 : 100 concentration in Dako antibody diluent and incubated for 4 hours. The BUB1B staining required a 4-minute treatment with Ventana Protease 1 prior to a 60-minute incubation with the BUB1B antibody (diluted 1 : 100, Abcam, #AB54894). The Ventana Omni Anti-Mouse HRP Secondary Antibody (prediluted) was used for 16 min. The detection system used was the Ventana Omni UltraMap kit, and slides were then counterstained with hematoxylin. Slides were dehydrated and cover-slipped as per standard tissue core laboratory protocol.

2.3. Control Tissues Used for Immunohistochemical Optimization and Test Runs. Positive control tissues that were used for optimization of the above IHC protocols included tonsillar lymphoid tissue for TOP2A and MCM2 and spleen for BUB1B protein, per manufacturer's recommendations. For negative controls, the respective primary antibodies were replaced by commercially available nonimmunized normal serum. Both types of controls showed satisfactory results.

2.4. Scoring of Immunohistochemical Expression of TOP2A and MCM2 Proteins. The stained slides were evaluated by the

Table 1: Clinicopathologic summary of IDC, molecularly high-risk and molecularly low-risk, histologically normal breast tissue specimens.

			Outside institution				Moffitt Cancer Center (MCC)								
Case no.	Tiisue core ID (frozen specimens for microarray)	Patient age (Years)	Side of breast sampled	Date of first-tissue diagnosis of BRCA	Surgical procedure at first-tissue diagnosis of BRCA; INA = Information not available	Pathologic diagnosis of BRCA	Date of surgical procedure at MCC	Side of breast sampled	Surgical procedure	Axillary LN dissection = AX; Sentinel LN dissection, axillary = SLN-AX; Not done = ND	Final pathologic diagnosis	Histologic grade (Invasive carcinoma)	Tumor size (cm)	DCIS	Other histopathologic findings (resected specimen)
1	X	41	Left	2001.04.10	Core biopsy	IDC, DCIS, high grade, comedo type	2005.08.04	Left	Lumpectomy	Intramammary lymph nodes	IDC	3	5	Present	X
1	X	41	Right	X	X	X	2005.08.04	Right	Lumpectomy	ND	IDC	3	4	Present	Skeletal muscle invasion, multifocal
2	X	38	Left	2005.07.01	Core biopsy	IDC	2005.08.03	Left	Modified radical mastectomy	SLN-AX	Papillary CA with a focus of IDC	3	7.5	Present	IDC focus 0.8 cm
3	X	68	Right	2005.07.20	INA	IDC	2005.08.09	Right	Total mastectomy	SLN-AX	IDC	3	2.2	Present	X
4	X	47	Left	2005.11.28	INA	IDC	2006.01.09	Left	Mastectomy	SLN-AX	IDC	3	2	Present	Focal ductal hyperplasia, radial scar, sclerosing adenosis, microcalcifications
4	X	47	Right	X	X	X	2006.01.09	Right	Mastectomy	SLN-AX	Fibroadenomatoid hyperplasia, florid ductal hyperplasia, sclerosing adenosis, microcalcifications	NA	NA	Absent	X
5	X	53	Right	2006.05.09	Core biopsy	IDC	2006.07.31	Right	Mastectomy	AX	Residual IDC	3	2.8	NS	involve dermis of skin/nipple
6	X	71	Left	2006.04.12	Core biopsy	Ductal & Lobular CA	2006.05.23	Left	Modified radical mastectomy	SLN-AX	IDC w/lobular features	3	4	Present	Tumor involves dermal angiolymphatic space in areola
7	1480	61	Right	2006.05.09	INA	IDC	2006.08.01	Right	Mastectomy	SLN-AX	Benign breast	NA	NA	Absent	ALH, proliferative fibrocystic changes
7	X	61	Left	X	X	X	2006.08.01	Left	Mastectomy	SLN-AX	IDC	2	2.6	Absent	
8	1481	86	Right	2006.07.31	Core biopsy	IDC	2006.08.29	Right	Total mastectomy	SLN-AX	IDC	3	8.5	Present	involve dermis of skin/nipple
9	1482	52	Left	2006.02.22	Core biopsy	IDC	2006.03.30	Left	Total mastectomy, skin-sparing	SLN-AX	IDC	2	1.5	Present	X
10	1483	80	Left	2005.06.13	Excisional biopsy	Mucinous Carcinoma	2005.08.16	Left	Total mastectomy	Not done	No residual carcinoma	NA	NA	Absent	X
11	1484	42	Left	2005.07.06	Core biopsy	DCIS solid type	2005.08.15	Left	Mastectomy	SLN-AX	Residual DCIS	NA	0.4	Present	DCIS, multifocal
11	X	42	Right	X	X	X	2005.08.15	Right	Mastectomy	SLN-AX	Benign breast	NA	NA	Absent	X

Table 1: Continued.

			Outside institution					Moffitt Cancer Center (MCC)							
Case no.	Tiisue core ID (frozen specimens for microarray)	Patient age (Years)	Side of breast sampled	Date of first-tissue diagnosis of BRCA	Surgical procedure at first-tissue diagnosis of BRCA; INA = Information not available	Pathologic diagnosis of BRCA	Date of surgical procedure at MCC	Side of breast sampled	Surgical procedure	Axillary LN dissection = AX; Sentinel LN dissection, axillary = SLN-AX; Not done = ND	Final pathologic diagnosis	Histologic grade (Invasive carcinoma)	Tumor size (cm)	DCIS	Other histopathologic findings (resected specimen)
12	1485	52	Left	2006.03.31	Core biopsy	IDC	2006.05.30	Left	Total mastectomy	ND	No residual carcinoma	NA	NA	Absent	X
12	X	52	Right	X	X	X	2006.05.30	Right	Rt modified radical prophylactic mastectomy	AX	Benign breast	NA	NA	Absent	X
13	1486	67	Right	2006.05.02	Core biopsy	DCIS	2006.07.12	Right	Mastectomy	SLN-AX	DCIS	NA	1.2	Present	X
13	X	67	Left	X	X	X	2006.07.12	Left	prophylactic mastectomy	SLN-AX	Benign breast	NA	NA	Absent	X
14	1487	67	Left	2006.06.12	Core biopsy	DCIS	2006.07.31	Left	Mastectomy	SLN-AX	No residual DCIS	NA	NA	Absent	
14	X	67	Right	X	X	X	2006.07.31	Right	prophylactic mastectomy	SLN-AX	Benign breast	NA	NA	Absent	microcalcifications
15	1488	49	Left	2005.05.23	Excisional biopsy	DCIS	2005.08.02	Left	Total Mastectomy	SLN-AX	Residual DCIS	NA	X	Present	X
16	1489	49	Left	2005.05.27	Excisional biopsy, subareolar	Adenoid cystic CA	2005.06.14	Left	Total Mastectomy	SLN-AX	No residual carcinoma	NA	NA	Absent	ADH
16	X	49	Right	X	X	X	2005.06.14	Right	prophylactic mastectomy	SLN-AX	Benign breast	NA	NA	Absent	X
17	1490	39	Right	2005.04.26	Excisional biopsy	DCIS solid type	2005.06.29	Right	Mastectomy	SLN-AX	DCIS, multifocal	NA	2.2	Present	X
18	1491	85	Left	2005.06.30	Excisional biopsy	Intracystic CA	2005.07.28	Left	Mastectomy	SLN-AX	No residual carcinoma	NA	NA	Absent	ADH
19	1492	47	Left	2005.11.11	Needle-loc excisional biopsy	IDC	2006.05.02	Left	Skin-sparing total mastectomy	SLN-AX	IDC focal micropapillary features	3	2.5	Present	X
19	X	47	Right	2006.02.27	Needle core biopsy	Fibrocystic changes	2006.05.02	Right	Needle-loc excisional biopsy	SLN-AX	Adenoma w/adenomyoepitheliomatous features and focal atypia	NA	NA	Absent	Sclerosing adenosis, cystic/apocrine changes
20	1493	55	Right	2006.01.03	Cytology: Adenocarcinoma	IDC	2006.02.20	Right	Mastectomy	SLN-AX	IDC	3	2	NS	X
20	X	55	Left	X	X	X	2006.02.20	Left	Mastectomy	SLN-AX	ALH; No invasive carcinoma	NA	X	X	ALH involving lactiferous duct
21	1494	50	Right	2006.03.15	Core biopsy	IDC	2006.05.22	Right	Bilateral nipple sparing mastectomy	SLN-AX	No residual carcinoma	NA	NA	Absent	X
21	X	50	Left	X	X	X	2006.05.22	Left	As above	SLN-AX	Benign breast	NA	NA	Absent	X
22	1495	18	Right	NA	NA	No clinical evidence of invasive carcinoma	2006.06.19	Right	prophylactic mastectomy*	SLN-AX	Benign breast	NA	NA	Absent	X
22	X	18	Left	X	X	X	2006.06.19	Left	prophylactic mastectomy*	SLN-AX	Benign breast	NA	NA	Absent	X
23	1496	56	Right	2006.04.04	Unknown	DCIS	2006.06.15	Right	Bilateral mastectomy	SLN-AX	No residual DCIS	NA	NA	Absent	Ductal hyperplasia, microcalcifications
23	X	56	Left	X	X	X	2006.06.15	Left	Bilateral mastectomy	SLN-AX	Benign breast	NA	NA	Absent	Fat necrosis, microcalcifications
24	1497	48	Right	2006.06.27	Lumpectomy	IDC, ILC	2006.07.28	Right	Modified radical mastectomy	SLN-AX	No residual invasive carcinoma; LCIS involves nipple duct	NA	NA	Absent	X
24	X	48	Left	X	X	X	2006.07.28	Left	prophylactic mastectomy	ND	Benign breast	NA	NA	Absent	Focal secretory change

* Strong family history of breast cancer and patient tested positive for BRCA1 gene.

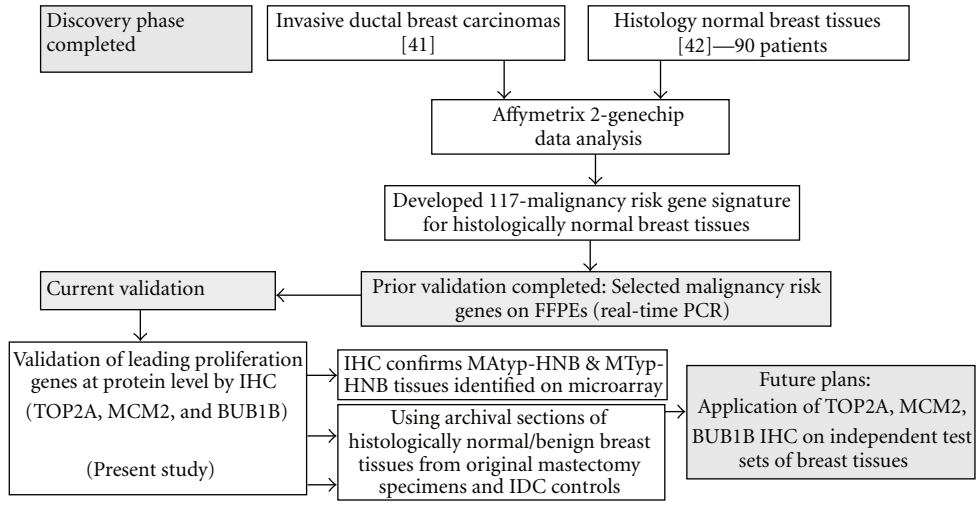


Figure 1: Molecular markers of malignancy in histologically normal breast tissues. Context and evolution of our prospective experimental plan.

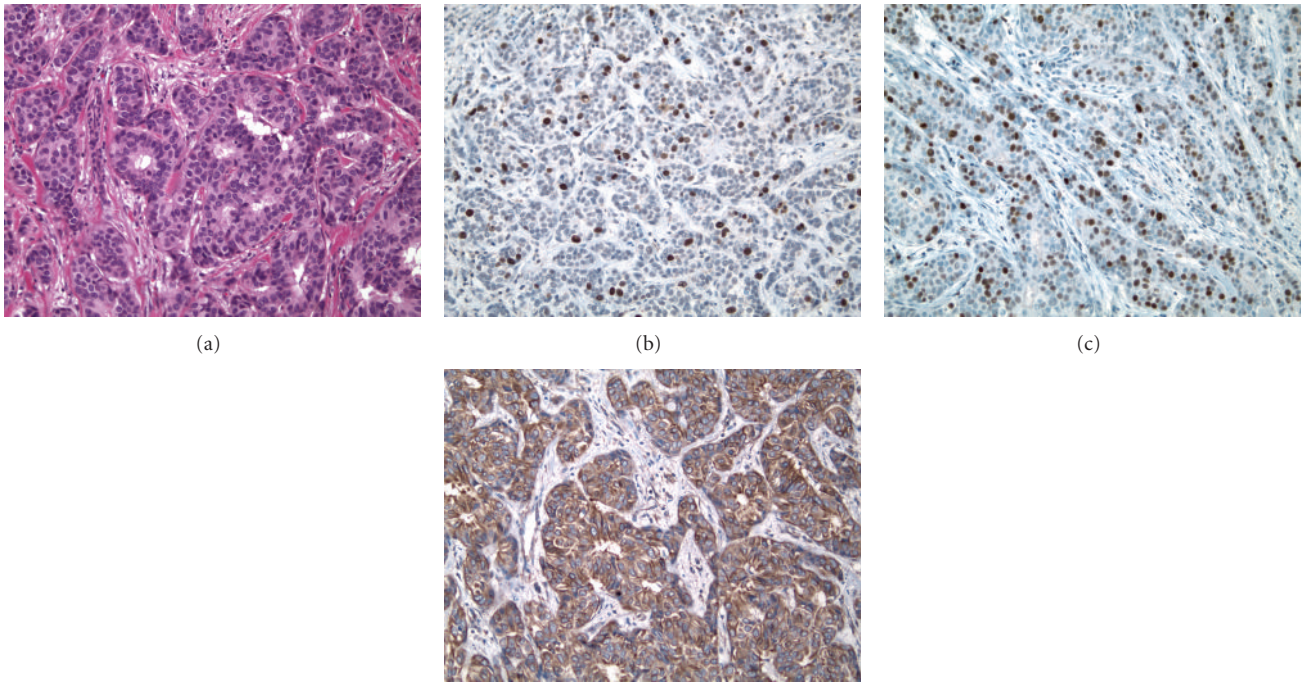
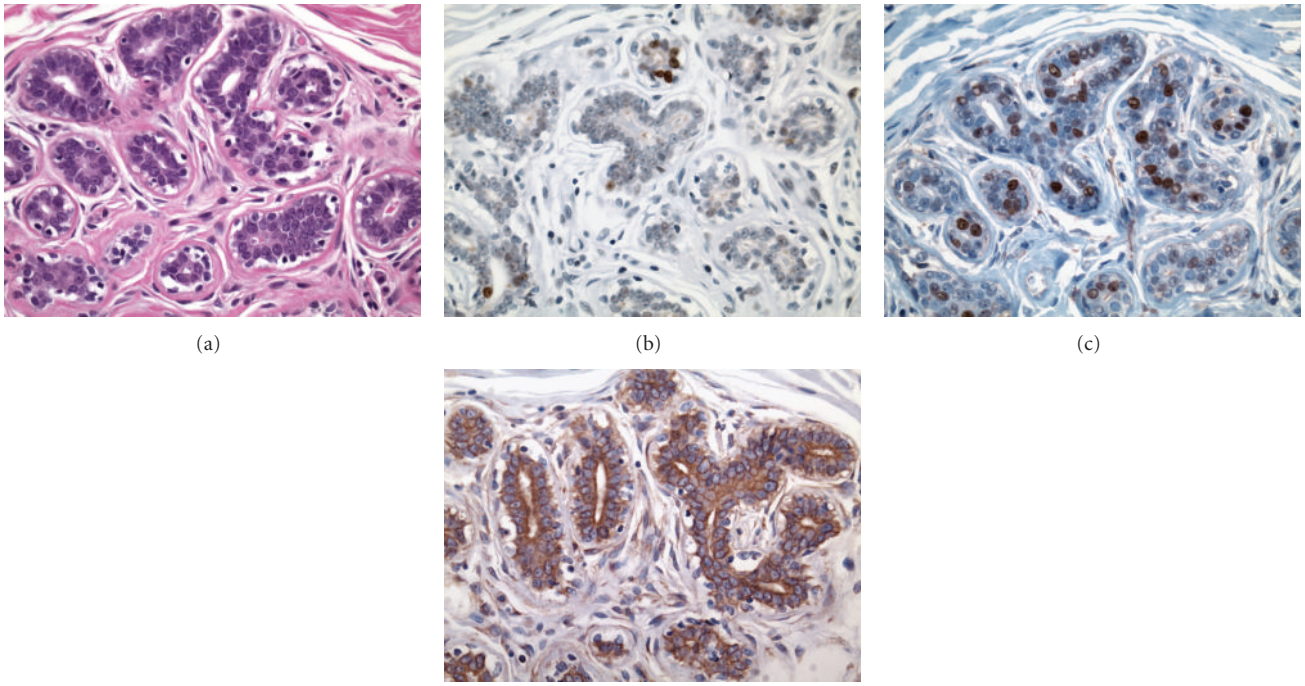


Figure 2: Serial archival sections representative of an IDC stained for H&E, TOP2A, MCM2 and BUB1B proteins. (a) Primary invasive ductal carcinoma (IDC) of the breast, grade 2, featuring focal tubular differentiation. (b, c, d) Distinct nuclear immunoreactivity for TOP2A marking the presence of cycling cells in about 15% of the infiltrating tumor cells, and for MCM2 marking the "licensed" population in about 1/3rd of the infiltrating tumor cells and diffuse cytoplasmic immunoreactivity (2+) with focal cell membrane accentuation for BUB1B protein (Immunoperoxidase staining (IMPOX staining); original magnifications 200x).

breast pathologist on the study with extensive experience in immunohistochemistry (AN). Immunohistochemical staining for TOP2A and MCM2 was localized to the nuclei of the tumor cells and the normal breast epithelium, while the expression of BUB1B protein was localized to the cytoplasm of the tumor and normal breast epithelial cells. In order to calculate TOP2A and MCM2 nuclear staining indices in IDC tissue sections, up to 2000 tumor cells and in the case of histologically normal breast tissues (HNB-HGMA and HNB-LGMA) tissue sections up to 500 nonneoplastic breast epithelial cells were evaluated by absolute counting of positive (stained) and negative (unstained) cells in each section. TOP2A and MCM2 indices were recorded as per cent positive nuclei as previously described [13]. As outlined



(a) (b) (c)

(d)

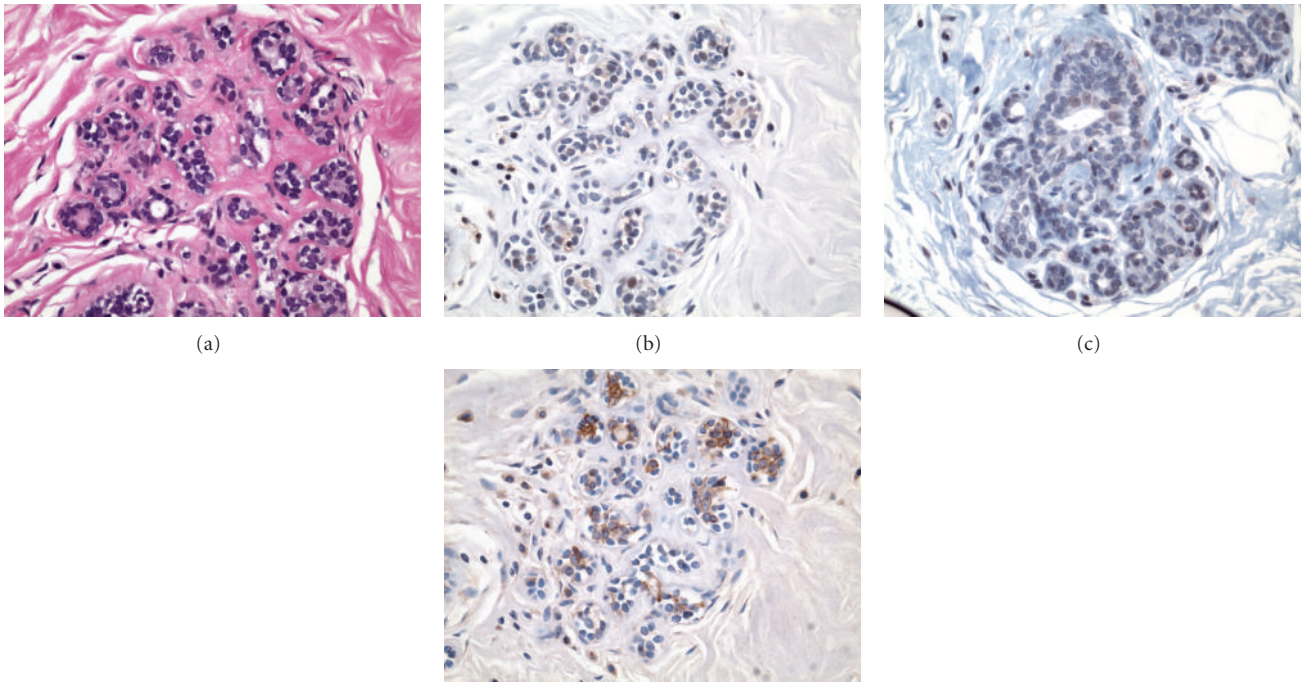
Figure 3: Serial archival sections representative of histologically normal breast tissues with high-grade molecular abnormality stained for H&E, TOP2A, MCM2 and BUB1B proteins. (a) Portion of a TDLU from a histologically normal breast tissue with high-grade molecular abnormality (Case 22, specimen 1495). Serial sections showing the same TDLU as in (a) with distinct nuclear immunoreactivity for TOP2A (b) and MCM2 (c) in the epithelial cell nuclei, and diffuse cytoplasmic immunoreactivity (2+) for BUB1B protein (d) in the mammary epithelial cells. (IMPOX staining; original magnifications 400x).

in the scheme published by Gonzalez et al. [14], these evaluations were made in the highest expression areas of the tumor and histologically normal breast tissues (Figures 2(b), 2(c), 3(b), 3(c), 4(b), and 4(c)).

In the IDCs, both TOP2A- and MCM2-positive tumor cells were often more frequent at the peripheral/advancing edge of the tumor mass (Figures 2(b) and 2(c)), while in HNB tissues such cells were more randomly distributed within the epithelial lining of the mammary acini and ducts (Figures 3(b), 3(c), 4(b), and 4(c)). Overall, expression of these markers was observed predominantly in the mammary epithelial cells. In some areas, nuclear staining was also noted in an occasional myoepithelial cell in the outer layers of the benign mammary acini and ducts. Since myoepithelial expression was not a consistent finding in most benign mammary lobules, it was not included in the determination of TOP2A and MCM2 index.

2.5. Scoring Immunohistochemical Expression of BUB1B Protein. Since the intensity of cytoplasmic staining and the percentage of epithelial cells stained for BUB1B protein was variable from case to case and from lobule to lobule within the same case, a comprehensive immunohistochemical scoring method (*H*-score method) [15] was used for semiquantitative evaluation of BUB1B protein expression in the entire tumor and normal breast tissue sections: BUB1B protein staining intensity in the malignant (IDC) or benign breast epithelial cells was scored 0 when there was no cytoplasmic staining, 1+ for weak, 2+ for intermediate, and 3+ for strong cytoplasmic staining. The products of stained epithelial cells (%) and the respective staining intensity (0, 1+, 2+, 3+) were added to calculate the total BUB1B protein immunohistochemical staining score (*H*-score) for each IDC tissue and for each histologically normal TDLU in the HNB tissue section evaluated (Figure 5). The total number of TDLUs evaluated for immunohistochemical expression of TOP2A, MCM2, and BUB1B proteins in the HNB-HGMA and the HNB-LGMA tissue sections ranged from 6 (no other FFPE section with greater # of TDLUs was found on review of all archival slides on that case) up to a maximum of 39 TDLUs/section (Figure 5). The average number of TDLUs evaluated per HNB tissue section was 31 (range 6 to 39 TDLUs per section) per HNB-HGMA tissue section analyzed and 24 (range of 17–35 TDLUs per section) per HNB-LGMA tissue section (Table 3). For most precise interpretation of immunoreactive nuclei, the sections were assessed using the 20x objective.

2.6. Differential Expression of TOP2A Protein in Independent Sets of Benign, Premalignant, and Cancerous Breast Tissues. Apart from cross-platform validation of 3 of our leading malignancy genes in archival HNB tissue samples, we further demonstrated the differential expression of TOP2A protein on independent test sets of Histologically normal breast



(a) (b) (c) (d)

Figure 4: Serial archival sections representative of a histologically normal breast tissues with low-grade molecular abnormality stained for H&E, TOP2A, MCM2 and BUB1B proteins. (a) Portion of a TDLU from a molecularly low-risk, histologically normal breast tissue (Case 8, specimen 1481). Serial sections showing the same TDLU as in (a) without any expression of TOP2A (b) and MCM2 (c) in the epithelial cell nuclei. There is a focal cytoplasmic immunoreactivity (1+ to 2+) for BUB1B protein (d) in some of the mammary epithelial cells in this field. (IMPOX staining; original magnifications 200x).

tissues, including reduction mammoplasty samples, benign breast tissue from patients with and without synchronous breast cancer, and a set of DCIS and invasive breast carcinomas in a custom-designed breast TMA (Figure 10).

2.7. Statistical Analysis. Analysis of variance was used to test the differences among the three sample groups (IDC, HNB-HGMA, and HNB-LGMA tissues) with the Tukey method to adjust for P value for pairwise comparison. This approach was used for analyzing the immunohistochemical expression data both from the FFPE sections and the breast TMA. Spearman correlation analysis was used to test the correlation between immunohistochemical expression of TOP2A, MCM2, and BUB1B proteins in the 3 sample groups.

3. Results

3.1. Patient Characteristics. The 18 histologically normal breast tissues with low-grade ($N = 9$) and high-grade ($N = 9$) molecular abnormalities were identified based on the differential expression of our breast malignancy genes from a total of 143 frozen normal breast tissue samples collected from mastectomies in patients with invasive breast carcinoma, DCIS, or prophylactic mastectomies (prior microarray experiment). We then summarized pertinent clinicopathologic characteristics of these patients with HNB tissues with low-grade molecular abnormalities (Cases 7–15) and those with high-grade molecular abnormality (Cases 16–24) (Table 1). Four of the nine patients whose HNB tissues showed low-grade molecular abnormality on microarray had the final pathologic diagnosis of IDC, 4 had only DCIS, and 1 had mucinous carcinoma. Of nine patients whose HNB tissues showed high-grade molecular abnormality on microarray, two patients had the final pathologic diagnosis of IDC, one tubular carcinoma, one adenoid cystic carcinoma, one invasive lobular carcinoma, one papillary intracystic carcinoma, 2 DCIS, and one patient had no histologic evidence of malignancy in the prophylactic mastectomy specimen, despite thorough sampling. The last patient underwent prophylactic bilateral mastectomy because of strong family history of breast cancer and had tested positive for the BRCA1 gene.

Mean age for the patient groups with IDCs, HNB-HGMA, and HNB-LGMA tissues was 63, 50, and 55 years, respectively (Table 2). Based on the analysis of variance (ANOVA), the difference in the distribution of patient ages at the time of diagnosis of their breast cancers (and collection of histologically normal tissues for the current analysis) was not statistically significant ($P = .29$). Since most patients whose normal breast tissues were found to exhibit HGMA or LGMA on prior microarray analysis [11] were perimenopausal, the differential expression of TOP2A, MCM2, and BUB1B proteins (proliferation gene products) in this

Table 2: Patient age distribution for IDC, molecularly high-risk and low-risk, histologically normal breast tissue groups.

	IDC patients	Patients with histologically normal breast tissues with low-grade molecular abnormality on microarray confirmed by IHC	Patients with histologically normal breast tissues with high-grade molecular abnormality on microarray confirmed by IHC
Mean age	63	55	50
Standard deviation	14.3	15.16	17.48
Total no. of cases	6	9	9

Table 3: Mean TOP2A and MCM2 indices and BUB1B protein expression scores in IDCs and molecularly high-risk and low-risk, histologically normal breast tissues.

Archival specimen type	Average no. of TDLUs evaluated/specimen (Range)	Mean TOP2A index (%) by IHC	Mean MCM2 index (%) by IHC	Mean BUB1B protein expression score (*H*-score) by IHC (Range)
IDCs ($N = 6$)	Not applicable	27 (15–35)	47 (30–80)	149 (80–200)
Histologically normal breast tissues with high-grade molecular abnormality ($N = 9$) on microarray	31 (6–39)	11 (2–30)	20 (8–35)	68 (33–113)
Histologically normal breast tissues with low-grade molecular abnormality ($N = 9$) on microarray	24 (17–35)	2 (1–3)	4 (1–12)	17 (10–22)
P value	.18	<.005	<.05	<.005

validation study is unlikely to be due to proliferative effect of estrogen on the normal/benign breast tissues analyzed.

3.2. TOP2A, MCM2, and BUB1B Protein Immunohistochemistry

3.2.1. Localization of Immunohistochemical Staining. TOP2A and MCM2 immunostaining was localized to the nuclei of the tumor cells (Figures 2(b) and 2(c)) and benign mammary epithelium (Figures 3(b) and 3(c)), while BUB1B protein immunostaining was cytoplasmic (Figures 2(d) and 3(d)), as has been demonstrated in a variety of normal human tissues [16]. In addition to cytoplasmic localization, an accentuation of BUB1B immunostaining (Figure 2(d)) was notable in cell membranes in some of the cases. Overall, a large proportion of tumor cells in the IDCs demonstrated a distinct nuclear staining for TOP2A (Figure 2(b) and MCM2 proteins (Figure 2(c)) and cytoplasmic staining for BUB1B protein (Figure 2(d)). However, the expression of these 3 biomarker proteins was found in smaller proportions of the epithelial cells lining the TDLUs present in the HNB-HGMA (Figures 3(b), 3(c), and 3(d)) and HNB-LGMA (Figures 4(b), 4(c), 4(d)) tissues analyzed.

3.2.2. TOP2A Protein Expression in IDCs and Histologically Normal Breast Tissues with High-Grade and Low-Grade Molecular Abnormality on Microarray. Expression of TOP2A was nuclear both in the tumor cells (Figure 2(b)) and in the acinar and ductal epithelial cells present in the histologically normal breast tissues with high-grade (Figure 3(b)) and low-grade (Figure 4(b)) molecular abnormality. Mean TOP2A nuclear staining index values for IDCs and histologically normal breast tissues with high-grade and low-grade molecular abnormality were 27, 11, and 2, respectively. Compared to HNB tissues with low-grade molecular abnormality, TOP2A expression in HNB tissues with high-grade molecular abnormality was significantly higher, both in terms of absolute (Table 4) and mean (Table 3, Figure 6) TOP2A expression indices, thus validating our TOP2A gene expression data from frozen to archival histologically normal breast tissues at the protein level.

MCM2 protein expression in IDCs and histologically normal breast tissues with high-grade and low-grade molecular abnormality on microarray Expression of MCM2 was nuclear both in the IDC cells (Figure 2(c)) and in the acinar and ductal epithelial cells present in the histologically normal breast tissues with high-grade (Figure 3(c)) and low-grade (Figure 4(c)) molecular abnormality. Mean MCM2 staining indexes for IDCs and histologically normal breast tissues with high-grade and low-grade molecular abnormality on microarray were 47, 20, and 4, respectively, showing higher immunohistochemical expression of MCM2 in the HNB tissues with high-grade molecular abnormalities compared to the HNB tissues with low-grade molecular abnormality (Table 4, Figure 7), thus validating the same trend as was evident in our gene expression data. While the majority of cases in HNB tissues with low-grade molecular abnormality had MCM2 index of 1-2%, 2 of the cases (Case #s 9 and 14) (Table 4) had higher MCM2 indices (12% and 8%, resp.), closer to the MCM2 index of some of the HNB tissues with high-grade molecular abnormality, suggesting that there may be a degree of heterogeneity in the expression of MCM2 protein in HNB tissues.

3.2.3. BUB1B Protein Expression in IDCs and Histologically Normal Breast Tissues with High-Grade and Low-Grade Molecular Abnormality on Microarray. Mean BUB1B protein

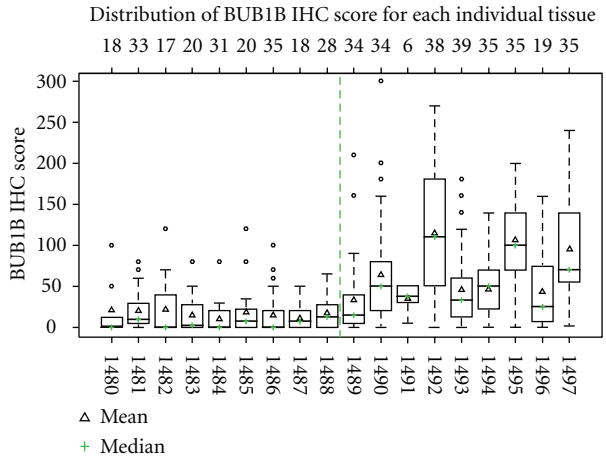


Figure 5: Whisker plot showing BUB1B protein expression scores for each individual histologically normal breast tissue with high-grade ($N = 9$) and low-grade molecular abnormality ($N = 9$) analyzed. The median BUB1B IHC score for each specimen is represented by horizontal lines and symbol +, while mean BUB1B IHC score is represented by Δ. Both mean and median expression scores for the HNB tissues with high-grade molecular abnormality on microarray (cases 1489–1497) are higher than those for the molecularly low-risk HNB tissues with low-grade abnormality on microarray (cases 1480–1488). Overall, there is a greater variation in the expression scores for the HNB tissues with high-grade molecular abnormality as compared to those with low-grade molecular abnormality (SD = 48.4 versus 24.8; $P = .003$). The top row reflects the number of TDLUs that were evaluated for IHC expression of BUB1B protein in the respective stained section, representing each histologically normal breast tissue specimen.

cytoplasmic staining scores for IDCs and histologically normal breast tissues with high-grade and low-grade molecular abnormality on microarray were 149, 68, and 17, respectively (Table 3). As compared to low-risk normal breast tissues, this pattern of significantly higher immunohistochemical expression of BUB1B protein in histologically normal breast tissues with high-grade molecular abnormality as compared to low-grade molecular abnormality on microarray confirms the gene expression trends observed on microarray, thus validating our BUB1B RNA expression data at the protein level. Figure 5 shows the distribution of expression of BUB1B protein in the two molecular sets of histologically normal breast tissues. The histologically normal breast tissues with high-grade molecular abnormality had greater number of TDLUs available for evaluation per individual BUB1B protein-stained section than the HNB tissues with low-grade molecular abnormality on microarray molecularly low-risk group (the average number of breast lobules evaluated was 31 versus 24, resp.), but this difference was not statistically different ($P = .18$).

3.2.4. Differential Expression of TOP2A, MCM2, and BUB1B Proteins in IDCs and Molecularly High-Risk and Low-Risk, Histologically Normal Breast Tissues. The immunohistochemical expression scores for TOP2A, MCM2, and BUB1B protein in the HNB-HGMA tissues were in the intermediate range between the higher scores (expression) for the IDCs and the lower scores (expression) for the HNB-LGMA tissues (Tables 3 and 4). In fact, for all 3 marker proteins, we observed a trend toward increasing immunohistochemical expression (TOP2A and MCM2 indices and BUB1B protein H-scores) from HNB-LGMA to HNB-HGMA tissues to the IDC tissues analyzed (Figures 6, 7, and 8). Analysis of variance showed that the differences in the immunohistochemical expression scores for TOP2A, MCM2, and BUB1B protein for the three types of tissues were statistically significant ($P < .005$ for TOP2A and BUB1B protein, and $P < .05$ for MCM2 for each pairwise comparison using the Tukey method). The differences in expression of these markers for individual pairs (and respective P values) are shown in Figures 6, 7, and 8. Furthermore, in comparing the HNB tissues with low-grade and high-grade molecular abnormality on microarray, the immunohistochemical expression of these 3 marker proteins was highly correlated (Spearman correlation ranges 0.84–0.90 with P value $< .0001$: $r = 0.84$ for TOP2A versus BUB1B, $r = 0.9$ for TOP2A versus MCM2, and $r = 0.88$ for BUB1B versus MCM2). Taken together, these results validate our microarray expression data for TOP2A, MCM2, and BUB1B at the protein level in archival histologically normal breast tissues.

3.2.5. Pathologic Characteristics of the Cases on Breast TMA Stained for TOP2A. In order to further validate the differential expression of TOP2A protein in various benign, atypical, premalignant, and cancerous breast tissues, we immunostained a breast TMA for TOP2A, using the same IHC protocol as outlined above. The various groups of breast lesions represented on this TMA were as follows.

Benign Lesions ($N = 15$). In this group seven adult females had undergone unilateral or bilateral reduction mammoplasty (RM). Others underwent diagnostic breast tissue sampling. Final pathologic evaluation showed histologically normal breast tissues with areas of benign breast disease (BBD) ($N = 10$), BBD with focal ductal hyperplasia (FDH) ($N = 2$), intraductal papilloma ($N = 1$), BBD with focus of atypical lobular hyperplasia (ALH) ($N = 1$), and BBD with focal fibroadenomatoid hyperplasia ($N = 1$).

Atypical Ductal Hyperplasia (ADH) without Invasive Breast Carcinoma ($N = 9$). All specimens in this group showed BBD with foci of ADH. In addition, six (66%) cases showed columnar cell change and four (44%) had atypical lobular hyperplasia. There was one case with pseudoangiomatous stromal hyperplasia (PASH) and one case with an intraductal papilloma.

ADH with Ipsilateral Invasive or In Situ Breast Carcinoma ($N = 8$). All of these cases showed ADH. In addition, three cases showed areas of invasive ductal carcinoma (IDC) while 5 cases had ductal carcinoma in situ (DCIS), 2 cases showed focal columnar cell change, and one of them also had an intraductal papilloma with atypia, a radial scar, and a fibroadenoma.

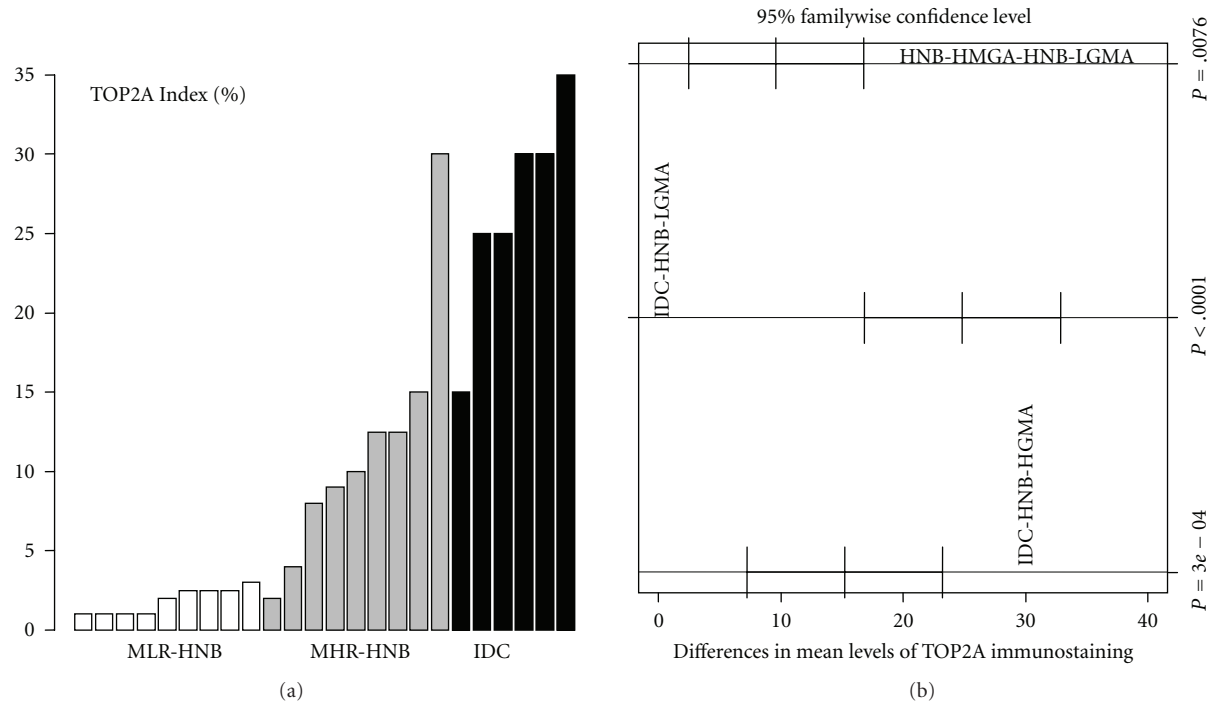


Figure 6: Immunohistochemical expresssion of TOP2A protein in HNB tissues with low-grade and high-grade molecular abnormalities and in IDCs. There is an obvious trend toward increasing expression from HNB tissues with low-grade molecular abnormality (white bars) to those with high-grade molecular abnormality (gray bars), and the IDCs (black bars), thus providing evidence for cross-platform validation of our original expression profiling data for TOP2A at the protein level. (a) Is the specimen-wise distribution of immunohistochemical expression of *TOP2A* for the HNB tissues with low-grade and high-grade molecular abnormality and IDC groups. (b) Is the pairwise comparison of TOP2A immunostaining among the three groups. For each comparison (e.g., IDC versus normal), a mean difference with a 95% confidence interval (95% CI) is displayed to examine whether the difference is statistically significant (A 95% CI deviated away from 0 is statistically significant). The adjusted *P* value for each comparison, based on Tukey method, is shown.

Ductal Carcinoma In Situ (DCIS) ($N = 15$). Of the fifteen specimens in this group, 14 (93%) were intermediate to high-nuclear grade DCIS and one low-nuclear grade DCIS. Among these two specimens had areas of adenosis, focal ductal hyperplasia, PASH, and a fibroadenoma in addition.

Invasive Ductal Breast Carcinomas ($N = 20$). These were histologically confirmed IDCs, of which 2 cases also had focal DCIS, intermediate to high nuclear grade. One IDC showed focal mucinous differentiation.

3.2.6. Differential Expression of TOP2A Protein in Benign, Atypical, and Premalignant, and Cancerous Breast Tissues. We found a striking trend toward increasing expression of TOP2A protein in this independent test set of histologically normal and benign breast tissues, ADH with or without synchronous invasive breast carcinoma, DCIS and invasive ductal breast carcinoma tissues, represented on the breast TMA. These results provide further validation of increasing expression of TOP2A protein along the histologic continuum of various breast lesions from benign to premalignant to invasive breast carcinomas it's (Figures 9(a), 9(b), 9(c), and 9(d)). For these specimen types, TOP2A protein expression data are summarized in Figure 10.

4. Discussion

There is increasing evidence to support the hypothesis that histologically normal breast tissues contain genetic and epigenetic abnormalities that render them more susceptible to neoplastic transformation and that they might be detected through molecular analyses. In patients with sporadic breast cancer, abnormalities of breast cancer susceptibility genes, including TP53, BRCA1, and BRCA2, have been identified in tumor tissue, and also in histologically normal TDLUs adjacent to carcinoma [17]. In a recent study, Larson et al. found a threefold increase in allelic imbalance (AI) in histologically normal breast tissue from sporadic breast cancer patients and BRCA1 gene mutation carriers as compared to women who underwent reduction mammoplasty [5], suggesting that these genetic abnormalities may be contributing to the risk of development of malignancy. More recently, altered telomeres and unbalanced allelic loci (markers of genetic instability) were found both in human breast cancers and in surrounding histologically normal breast tissues [7]. These findings provide further support to the "cancer field effect" concept recognizing the presence of genetically aberrant cells that may represent high risk cell populations within the histologically normal breast tissues. In a more recent study, [10] elucidated the molecular differences between histologically

Table 4: TOP2A, MCM2, and BUB1B protein expression scores in IDCs, molecularly low-risk and molecularly high risk, histologically normal breast tissues.

Case no.	Breast tissue specimen category (based on gene expression profiling)	Histologic tumor type on initial biopsy/lumpectomy	Final pathologic diagnosis on mastectomy	Histopathologic findings on archival tissue sections selected for IHC validation	Topoisomerase II-alpha (TOP2A) index (%)	MCM2-index (%)	BUB1B protein expression score (*H*-score)
1	Carcinoma	IDC	IDC, DCIS	Invasive Cancer	25	30	170
2	Carcinoma	IDC	Invasive papillary CA with a focus of IDC	Invasive Cancer	30	30	135
3	Carcinoma	IDC	IDC	Invasive cancer	15	80	145
4	Carcinoma	IDC	IDC	Invasive Cancer	35	60	80
5	Carcinoma	IDC	IDC	Invasive Cancer	25	50	165
6	Carcinoma	IDC, ILC	IDC w/ lobular features	Invasive cancer	30	30	200
HNB tissues with low-grade molecular abnormality (HNB-LGMA)							
7	HNB-LGMA 1	IDC	IDC	Benign breast tissue	2.5	2	21
8	HNB-LGMA 2	IDC	IDC	Benign breast tissue	2.5	6	20
9	HNB-LGMA 3	IDC	IDC	Benign breast tissue	2.5	12	22
10	HNB-LGMA 4	Mucinous carcinoma	No residual mucinous carcinoma	Benign breast tissue	1	2	15
11	HNB-LGMA 5	DCIS	Residual DCIS, multifocal	Benign breast tissue	1	1	10
12	HNB-LGMA 6	IDC	No residual IDC	Benign breast tissue	1	1	18
13	HNB-LGMA 7	DCIS	Residual DCIS	Benign breast tissue	2	2	15
14	HNB-LGMA 8	DCIS	No residual DCIS	Benign breast tissue	3	8	11
15	HNB-LGMA 9	DCIS	Residual DCIS	Benign breast tissue	1	1	17
HNB tissues with high-grade molecular abnormality (HNB-HGMA)							
16	HNB-HGMA 1	Adenoid cystic carcinoma	No residual adenoid cystic carcinoma	Benign breast tissue	8	12	33
17	HNB-HGMA 2	DCIS	DCIS, multifocal	Benign breast tissue	12.5	20	64
18	HNB-HGMA 3	Intracystic carcinoma	No residual intracystic carcinoma	Benign breast tissue	12.5	8	35
19	HNB-HGMA 4	IDC	IDC focal papillary features	Benign breast tissue	9	30	113
20	HNB-HGMA 5	IDC	IDC	Benign breast tissue	2	12	46
21	HNB-HGMA 6	IDC	No residual IDC	Benign breast tissue	15	30	46
22	HNB-HGMA 7	No prior biosy performed	Benign breast tissue-patient BRCA1+, strong family history of BC	Benign breast tissue	10	25	106

Table 4: Continued.

Case no.	Breast tissue specimen category (based on gene expression profiling)	Histologic tumor type on initial biopsy/lumpectomy	Final pathologic diagnosis on mastectomy	Histopathologic findings on archival tissue sections selected for IHC validation	Topoisomerase II-alpha (TOP2A) index (%)	MCM2-index (%)	BUB1B protein expression score (*H*-score)
23	HNB-HGMA 8	DCIS	No residual DCIS	Benign breast tissue	4	12	43
24	HNB-HGMA 9	IDC, ILC	No residual invasive carcinoma	Benign breast tissue	30	35	94

normal breast tissue from breast cancer patients and reduction mammoplasty controls and found a number of global gene expression abnormalities in the HNB tissues [10].

Using specific epigenetic biomarkers, we have previously mapped a number of DNA methylation changes in histologically normal breast tissues as a potential explanation as to why histologically normal breast tissues are at risk for local recurrence after surgical therapy for breast cancer [6]. We recently developed a 117-gene signature by comparing the gene expression profiles of a large prospective cohort of frozen invasive ductal breast carcinoma (IDC) and histologically normal breast tissues (HNB) from breast cancer patients [11]. This signature was first cross-validated on HNB tissues using qPCR including external validation on previously published datasets [11]. We then used our 117-gene malignancy signature to classify eighteen histologically normal breast tissues with high-grade and low-grade molecular abnormality, based on the level of expression of our top malignancy genes. The leading candidate genes in our malignancy-risk signature were proliferation genes, including TOP2A, MCM2, and BUB1B.

Here we present the results of cross-platform immunohistochemical validation of these candidate malignancy gene products (TOP2A, MCM2, and BUB1B proteins) on archival histological normal breast tissue sections from the mastectomies of the two patient groups in the original microarray experiment (those with HNB tissues with high-grade and low-grade molecular abnormalities). These candidate biomarkers were selected for validation based on the gene expression data and the availability of commercially available antibodies and to further investigate their usefulness as biomarkers of molecular abnormalities in histologically normal and benign breast tissues. We further confirmed the increasing expression of one of our malignancy-risk gene products in the present analysis on independent test sets of histologically normal breast tissues including reduction mammoplasty samples, which mostly represent the specimens with lowest risk of breast malignancy, histologically normal/benign breast tissues from patients with and without synchronous breast cancer and a set of DCIS and invasive breast carcinomas (IDCs) using a custom-designed breast TMA (Figure 10).

One of our leading malignancy risk genes identified on microarray analysis of the histologically normal breast tissues was topoisomerase II alpha (TOP2A). TOP2A is a key enzyme in regulating various chromosomal events during tumor cell replication. It is one of the markers of cell proliferation in human breast cancer [18]. It is also the molecular target for topo II-inhibitors, including anthracyclines (doxorubicin, epirubicin, daunorubicin, idarubicin), epipodophyllotoxins (etoposide, teniposide), actinomycin, mitoxantrone, and others [19–21]. The TOP2A gene is located adjacent to the HER-2 oncogene at the chromosome location 17q12q21 and is either amplified or deleted in breast cancer, with or without HER-2 amplification. Recent evidence suggests that amplification or deletion of TOP2A gene may account for sensitivity or resistance to *topo II*-inhibitor (anthracycline) therapy in breast cancer [21]. However, little is known about the role of TOP2A in histologically normal/benign breast tissues.

We identified TOP2A as a part of the malignancy-risk signature on our microarray experiment and, in this study, validated its expression at protein level as a potential biomarker of risk of malignancy in histologically normal breast tissues. In the archival sections from histologically normal breast tissues with high-grade and low-grade molecular abnormalities, we evaluated a large number of "morphologically normal TDLUs" and found that the level of expression of TOP2A protein in HNB tissues with high-grade molecular abnormality on microarray was intermediate between the expression levels in the HNB tissues with low-grade molecular abnormality on microarray and invasive ductal breast carcinoma tissues analyzed. Furthermore, the differences in the TOP2A expression levels between the two subsets of molecularly abnormal histologically normal breast tissues and IDCs were statistically significant. Since amplification of TOP2A gene leads to the overexpression of the TOP2A protein and better response to anthracycline therapy [22], while deletion of TOP2A gene leads to marked reduction in the expression of TOP2A protein and primary chemoresistance to TOP2 inhibitor drugs [23], our findings in histologically normal breast tissues, if clinically validated in larger series of histologically normal and benign breast tissues, may have potential implications for future chemopreventive trials in patients with various atypical and pre-malignant breast lesions.

Since TOP2A amplified tumor cells tend to be sensitive to topo-II inhibitor therapy while TOP2A deleted tumor cells tend to be resistant to anthracycline chemotherapy [21], the overall response of a given breast cancer case will depend on

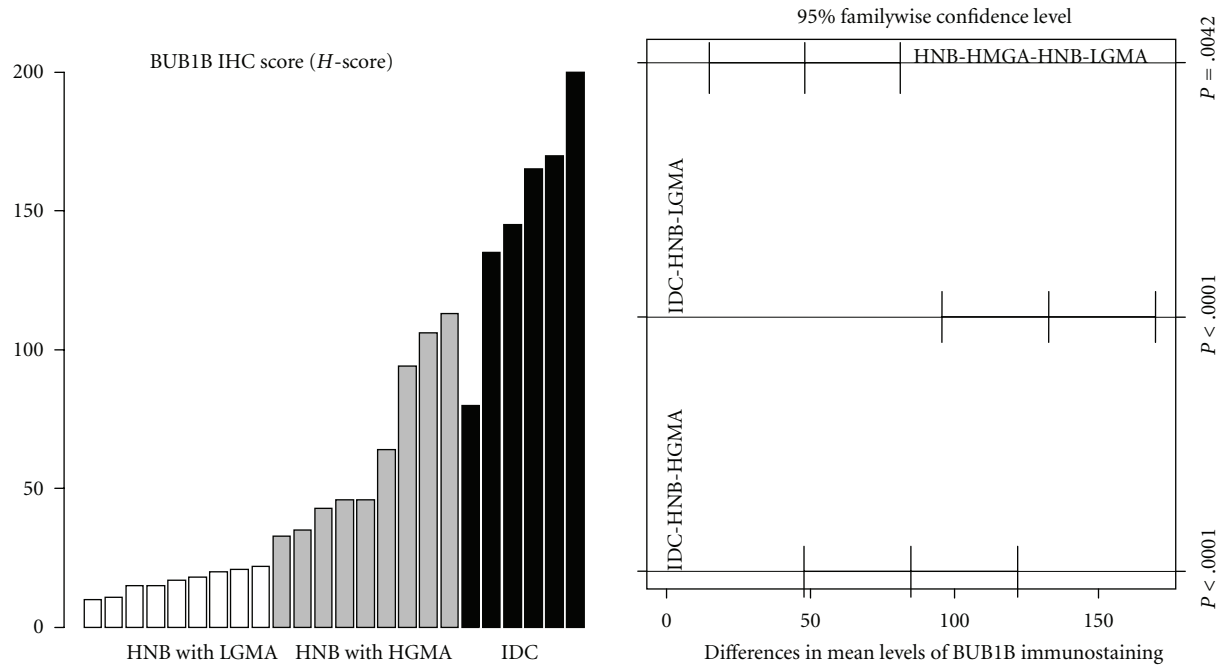


Figure 7: Immunohistochemical expression of BUB1B protein in HNB tissues with low-grade and high-grade molecular abnormalities and in IDCs. There is an obvious trend toward increasing expression from HNB tissues with low-grade molecular abnormality (white bars) to those with high-grade molecular abnormality (gray bars), and the IDCs (black bars), thus providing evidence for cross-platform validation of our original expression profiling data for BUB1B at the protein level. (a) Is the specimen-wise distribution of immuno-histochemical expression of *BUB1B* for the HNB tissues with low-grade and high-grade molecular abnormality and IDC groups. (b) Is the pairwise comparison of BUB1B immunostaining among the three groups. The adjusted P value for each comparison, based on Tukey method, is shown on (b).

the relative proportions of the 2 cell types. Furthermore, in locally advanced breast cancer, TOP2A levels in the primary tumor have been associated with greater tumor response to anthracycline therapy. It is, therefore, conceivable that in the case of molecularly abnormal histologically normal breast tissues increased expression of TOP2A may not only serve as a molecular biomarker of malignancy, but may also be potentially predictive of chemosensitivity to TOP2A inhibitors, in order to repress proliferation and subsequent transformation. These aspects merit further investigation on larger series of histologically normal and benign breast tissues.

MCM family of proteins are a novel class of proliferation markers, of which MCM2 is part of the prereplicative complex (pre-RC) that is assembled at the site of future DNA replication during the G1 phase to allow genome replication in the subsequent S phase. High-MCM2 index has been shown to correlate with high Ki-67 labeling [24] and has been shown to be a prognostic marker in a variety of human malignancies, including cancers of the esophagus, prostate, stomach and in diffuse large B-cell lymphoma [24–28]. In breast cancers, it appears to be a strong independent prognostic marker and the degree of MCM2 immunoreactivity has been correlated with high histologic grade [14, 29, 30]. In normal breast epithelium MCM2 has been shown to be a more sensitive marker of proliferation than the widely used proliferation marker, Ki-67 [14, 31], since it stains both the cycling cells and also the noncycling cells with proliferative potential [32]. However, not much is known regarding the association between MCM2 expression in normal and benign breast tissues.

In our malignancy-gene signature, MCM2 was one of the leading malignancy-associated genes in a set of histologically normal breast tissues from peri-menopausal beast cancer patients. In this study, using the standard immunohistochemical approach, we have observed that the MCM2 index in HNB tissues with high-grade molecular abnormality was in the intermediate range between IDCs and HNB tissues with low-grade molecular abnormality, thus validating the overexpression of MCM2 protein in the set of HNB tissues that were showed high-grade molecular abnormality on our original microarray data analysis.

In this study, we found expression of MCM2 protein in all of our cases of histologically normal breast tissues. Considering all of our normal breast samples together, the observed MCM2 index ranged from 1% to 35%. This wider variation is a reflection of an inherent case selection bias in our study, since we selected the 2 subsets of histologically normal breast tissues (with high- and low-grade molecular abnormality) based on differential expression of our malignancy- (proliferation-) associated genes. In a set of normal breast tissues from reduction mammoplasties,

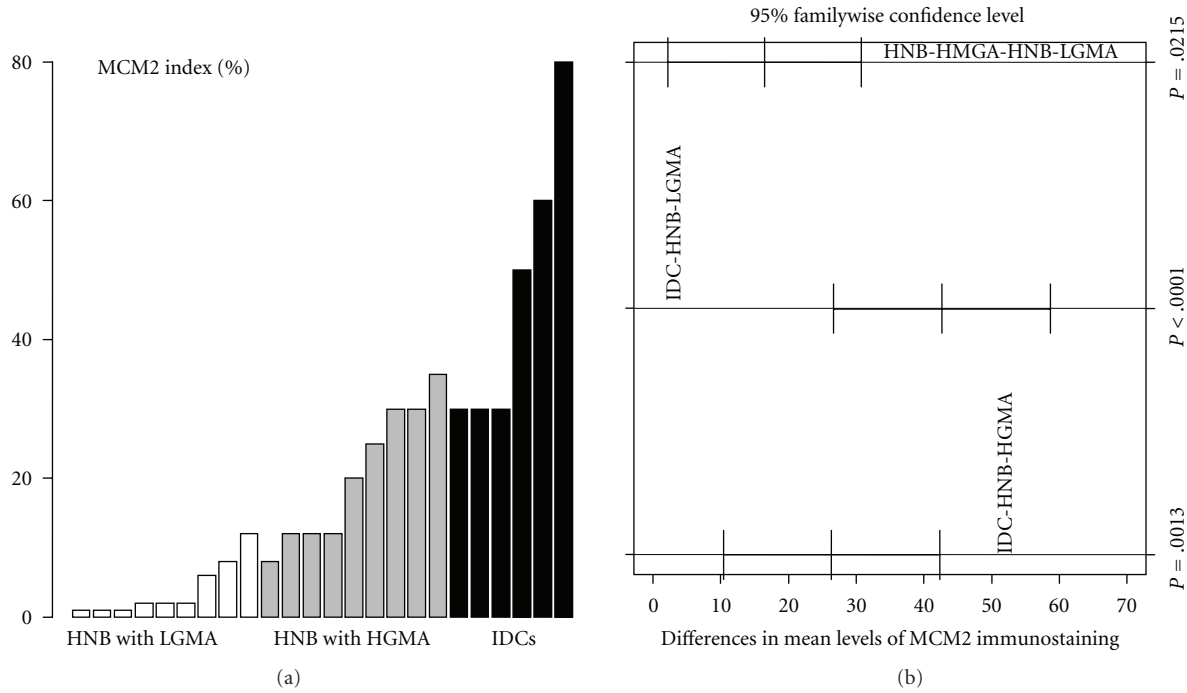
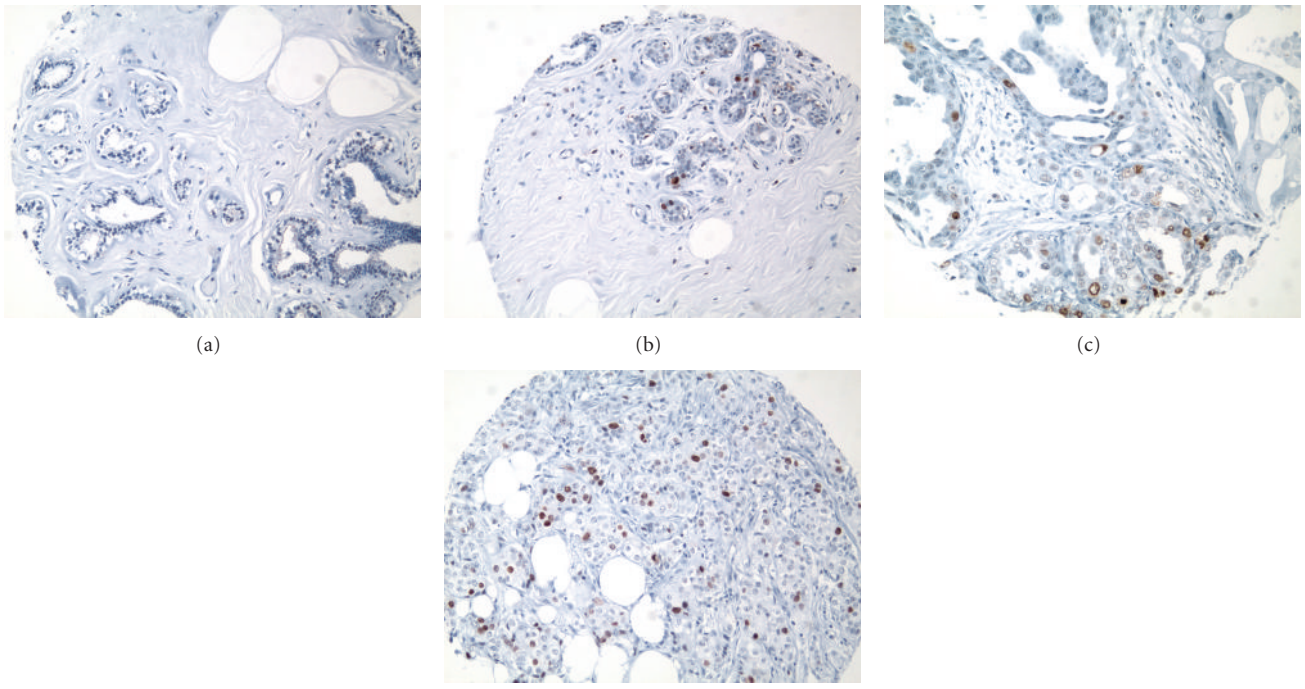


Figure 8: Immunohistochemical expresssion of MCM2 protein in HNB tissues with low-grade and high-grade molecular abnormalities and in IDCs. There is an obvious trend toward increasing expression from HNB tissues with low-grade molecular abnormality (white bars) to those with high-grade molecular abnormality (gray bars), and the IDCs (black bars), thus providing evidence for cross-platform validation of our original expression profiling data for MCM2 at the protein level. (a) Is the specimen-wise distribution of immunohistochemical expression of MCM2 for the HNB tissues with low-grade and high-grade molecular abnormality and IDC groups. (b) Is the pairwise comparison of MCM2 immunostaining among the three groups. The adjusted P value for each comparison, based on Tukey method, is shown on (b).

Shetty et al. found a median MCM2 expression of 35% [31]. This high level of expression is comparable to the highest levels of MCM2 expression in the HNB tissues with high-grade molecular abnormality in our study. Although normal breast tissues in the above study [31] were from the lowest risk specimens (reduction mammoplasties), a probable explanation for higher MCM2 indices in their study was premenopausal status of their patients, since estrogens are known to be a major promoter of proliferation in normal breast epithelium [33]. On the contrary, in our study it is unlikely that the higher MCM2 and other proliferation biomarkers (TOP2A and BUB1B) in the molecularly abnormal breast tissue samples were due to hormonal (estrogen) milieu of the patients studied, since both sets of HNB tissues (with high- and low-grade molecular abnormalities) in our study were from perimenopausal patients without any significant statistical difference in their ages. Therefore, a higher MCM2 expression in histologically normal breast tissues in our study is most likely a true molecular biomarker of malignancy rather than an estrogen-driven phenomenon.

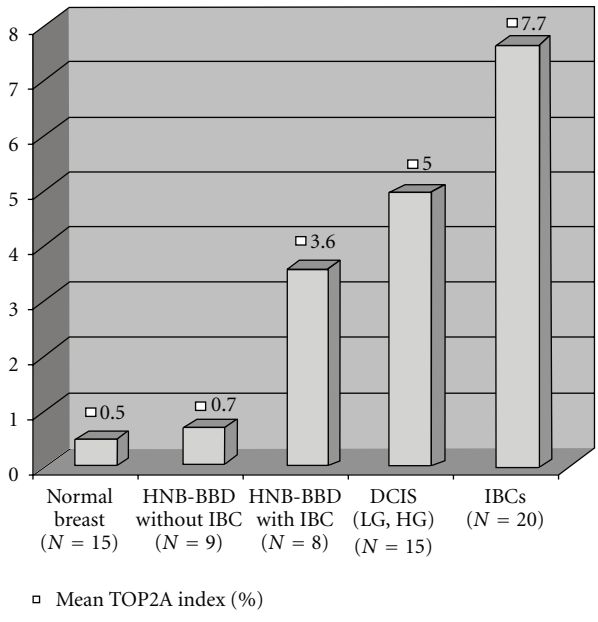
In another recent study of benign breast tissues from 30 patients who underwent lumpectomy for fibrocystic changes, ductal hyperplasia, and fibroadenomas, the overall MCM2 labeling index was from 0% to 12% [30]. This pattern of expression is comparable to the HNB tissues with low-grade moleculari abnormality in our study. In our preliminary analysis, we did not find an obvious and linear relationship between the expression of MCM2 and the histologically defined risk categories of benign breast disease. Interestingly, we found higher expression of MCM2 and other proliferation marker proteins in histologically normal TDLUs as compared to the adjacent hyperplastic lobular units and incidental areas of epithelial hyperplasia on the same histologic sections of HNB tissues. This suggests that the expression of our malignancy-associated proliferation marker proteins may be independent of the various histologic risk categories of benign breast disease as was originally defined on the basis of degree of epithelial proliferation and cytologic atypia [34–36], and subsequently endorsed at a Consensus Conference of the College of American Pathologists [37]. We are intrigued by this finding and would like to extend this into a systematic analysis of the expression of these biomarkers and various benign and preneoplastic histologic correlates of breast cancer risk, as they have been recognized in the literature over the years [35, 38–42].

BUB1B protein is a mitotic checkpoint kinase required for cell mitotic divisions following severe cell damage or mutation [43, 44]. It has been associated with cell proliferation both in neoplastic and nonneoplastic tissues [16, 45, 46] and also with tumor progression [47, 48]. BUB1B is also a cellular target of synuclein-gamma (SNCG, also



(a) (b) (c) (d)

Figure 9: Immunohistochemical expression of TOP2A protein. (a) Histologically normal breast tissue from a reduction mammoplasty (RM) case featuring lack of nuclear expression of TOP2A in the epithelial cells lining a normal TDLU. (b) Histologically normal breast tissue from a patient with synchronous breast cancer showing positive nuclear staining in 4-5% of the mammary epithelial cells-higher TOP2A expression than the HNB tissues from a reduction mammoplasty case illustrated in (a). (c-d) A larger proportion of epithelial cells are immunoreactive for nuclear TOP2A protein in ductal carcinoma in situ (DCIS) and in the invasive ductal carcinoma (IDC) infiltrating the mammary fat. These cases illustrate an obvious increase in TOP2A protein expression from the lowest risk specimen from a reduction mammoplasty case (a), to the higher-risk specimens (c) and (d) (IMPOX staining for TOP2A; original magnifications 200x).



(a)

Microscopic diagnosis	Mean TOP2A index (%)
Normal breast ($N = 15$)	0.5
HNB-BBD without IBC ($N = 9$)	0.7
HNB-BBD with IBC ($N = 8$)	3.6
DCIS (LG, HG) ($N = 15$)	5
IBCs ($N = 20$)	7.7

(b)

Figure 10: Mean TOP2A index in independent test sets of histologically normal breast (including reduction mammoplasty tissues), histologically normal and benign breast tissues from patients without and with synchronous cancer, DCIS and invasive breast carcinoma tissues. There is an obvious trend toward increasing TOP2A expression from benign to malignant breast tissues.

known as breast cancer specific gene 1), with which it may interact to inactivate the mitotic checkpoint, and contribute to resistance of beast cancer cells to microtubule inhibitors. Recently, a strong association has been found between BUB1B and other mitotic checkpoint genes and breast cancer risk [49]. Furthermore, checkpoint genes, including BUB1B, are expressed at high levels in breast cancer, both at transcriptional (RNA) and translational (protein) levels [50].

In this study, we have validated overexpression of BUB1B protein in histologically normal breast tissues that were found to be molecularly abnormal on microarray, thus validating our prior microarray and real-time PCR results. Our study suggests that BUB1B overexpression may be a new immunohistochemical biomarker of malignancy in histologically normal breast tissues. It will also be interesting to investigate the role of BUB1B overexpression as a potential therapeutic target for microtubule inhibitors and an immunohistochemical biomarker of predictive of chemosensitivity of atypical and pre-malignant breast lesions to these antimitotic agents.

Expression of hormone receptors is an established predictor of response of breast cancer to hormonal therapy in breast cancer, but markers predictive of chemosensitivity of breast cancer are less well defined [51]. In addition, markers that could predict effective prevention of human breast cancer in high-risk patient populations are largely unknown. Among the proliferation-associated proteins (TOP2A, MCM2, and BUB1B) that we have studied immunohistochemically on a set of IDCs and validated as immunohistochemical biomarkers of malignancy in histologically normal breast tissues, TOP2A and BUB1B protein are also known targets of established chemotherapeutic approaches in breast cancer: anthracyclines and antimicrotubule therapies, respectively. It will, therefore, also be interesting to explore how these biomarkers can be utilized as predictors of breast cancer response to TOP2A and antimicrotubule inhibitors.

5. Summary

To our knowledge, this is the first IHC-based analysis focusing on the pattern of coexpression of newer proliferation-associated proteins (TOP2A, MCM2, and BUB1B) in histologically normal breast tissues. In continuation of our prior transcriptional validation using qPCR, in this immunohistochemical validation study, we have demonstrated significantly higher expression of TOP2A, MCM2, and BUB1B proteins in a set of histologically normal breast tissues that were found to have high-grade molecular abnormality on microarray, based on our novel 117-gene malignancy signature. Taken together, these data further validate our leading candidate malignancy-risk genes (TOP2A, MCM2, and BUB1B) at the protein level. In addition, we have shown incremental expression of TOP2A protein on independent test sets of histologically normal breast tissues (including reduction mammoplasty samples), histologically normal/benign breast tissue from patients with and without synchronous breast cancer, and a set of DCIS and invasive breast carcinomas using a custom breast TMA. This study reveals new information about the coexpression of TOP2A, MCM2, and BUB1B proteins in histologically normal breast tissues and provide preliminary evidence to support further analyses of these proteins on larger series of histologically normal, benign, pre-malignant, and malignant breast tissues. Specifically, determination of TOP2A, MCM2, and BUB1B protein expression status may provide an objective tool to evaluate of the molecular signature of malignancy in histologically normal and benign breast tissues.

The immunohistochemical approach used here offered some distinct technical advantages over other techniques like qPCR or microarray: (1) combined assessment of the degree of expression (high versus low), microanatomical distribution (diffuse versus patchy), tissue (epithelial versus stromal), and subcellular (nuclear versus cytoplasmic) localization of the biomarker proteins in a given sample; (2) comparative evaluation of the relative expression of these marker proteins in histologically normal TDLUs and various incidental benign and pre-malignant breast lesions present in the same archival breast tissue sections. We do recognize one of the limitations of our study—the fewer numbers of histologically normal breast tissue analyzed. However, since we have successfully validated the expression of TOP2A, MCM2, and BUB1B proteins in HNB tissues with various grades of molecular abnormalities, we are in the process of now expanding our investigation to larger sample size and a wider range of benign pre-malignant and malignant breast tissues.

6. Conclusions

The data presented in this technical validation study of a novel set of molecular biomarkers (TOP2A, MCM2, and BUB1B proteins) in histologically normal breast tissues confirms our microarray data at the protein level. We have also unraveled a preliminary association between the expression of these marker proteins and different stages of mammary carcinogenesis (histologically normal to benign to pre-malignant and fully invasive malignant breast tissues). Additional studies on larger selection of histologically normal, benign, and pre-malignant breast tissues are needed to fully explore the clinical utility of these biomarkers in the stratification of histologically normal breast and benign and premalignant breast lesions into those with various levels of molecular abnormalities. Such classification may potentially be predictive of response of various benign, atypical, and pre-malignant to targeted chemopreventive approaches.

Abbreviations

TOP2A: Topoisomerase 2 alpha
MCM2: Minichromosome maintenance protein 2
BUB1B: Benzimidazoles 1 homolog beta'
HNB: Histologically normal breast
TDLUs: Terminal duct lobular units
BB: Benign breast
HELUs: Hyperplastic enlarged lobular units
EH: Epithelial hyperplasia

FDH: Focal ductal hyperplasia
ADH: Atypical ductal hyperplasia
DCIS: Ductal carcinoma in situ
BC: Breast cancer
IDC: Invasive ductal carcinoma
TMA: Tissue microarray
qPCR: Real-time PCR
HNB-HGMA: Histologically normal breast with high-grade molecular abnormality
HNB-LGMA: Histologically normal breast with low-grade molecular abnormality
FFPE: Formalin fixed, paraffin-embedded
SLN-AX: Sentinel lymph node dissection, AXILLARY
Sd: Standard deviation.

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Future Directions

In the future, the authors would like to evaluate the expression of TOP2A, MCM2, and BUB1B on larger clinical validation sample sets of breast tissues, in order to correlate the immunohistochemical expression of these biomarkers with future risk of development of malignancy and subsequent recurrence.

Conflict of Interests

The authors have no conflict of interests to disclose.

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Review Article

PAR Genes: Molecular Probes to Pathological Assessment in Breast Cancer Progression

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Taking the issue of tumor categorization a step forward and establish molecular imprints to accompany histopathological assessment is a challenging task. This is important since often patients with similar clinical and pathological tumors may respond differently to a given treatment. Protease-activated receptor-$_1$ (PAR_1), a G protein-coupled receptor (GPCR), is the first member of the mammalian PAR family consisting of four genes. PAR_1 and PAR_2 play a central role in breast cancer. The release of N-terminal peptides during activation and the exposure of a cryptic internal ligand in PARs, endow these receptors with the opportunity to serve as a "*mirror-image*" index reflecting the level of cell surface $PAR_{1\&2}$-in body fluids. It is possible to use the levels of PAR-released peptide in patients and accordingly determine the choice of treatment. We have both identified PAR_1 C-tail as a scaffold site for the immobilization of signaling partners, and the critical minimal binding site. This binding region may be used for future therapeutic modalities in breast cancer, since abrogation of the binding inhibits PAR_1 induced breast cancer. Altogether, both PAR_1 and PAR_2 may serve as molecular probes for breast cancer diagnosis and valuable targets for therapy.

1. Introduction

The classification of a tumor differentiation level is routinely based on histopathological criteria whereby poorly differentiated tumors generally exhibit the worst prognoses. However, the underlying molecular pathways that regulate the level of breast tumor development are as yet poorly described. Until now the pathological tissue criteria that entail tissue traits have not been defined by an appropriate set of genes. A challenging task is to take the issue of breast tumor categorization a step forward and establish molecular imprints to accompany histopathological assessment. This is important since often patients with similar clinical and pathological tumors may have a markedly different outcome in response to a given treatment. These differences are encoded by and stem from the tumor genetic profile [1]. Individual gene signature may complement or replace the traditional pathological assessment in evaluating tumor behavior and risk. This is the basis for optimizing our approach to personalized care whereby genomic finger prints may refine the prediction of the course of disease and the response to treatment [2]. Oncotype Dx is a clinically validated and widely used multigene assay (there are also other commercially available gene panels such as Mammaprint; Agendia Amsterdam, Netherland, and THEROS H/I; Biotheranostics, San Diego, CA), that quantifies the likelihood of breast cancer recurrence. This gene profile has been developed specifically for women with hormone receptor-positive (estrogen and progesterone receptor; ER, PR) and lymph node-negative disease. The gene profile consists of 21 genes that are associated with disease recurrence. Sixteen are cancer-related genes and 5 serve as reference genes. This gene panel is used to calculate the recurrence score (RS), a number that correlates with the specific likelihood of breast cancer recurrence within 10 years from the original diagnosis. Therefore, an ongoing goal is to identify important genes that play a central part in breast cancer biology and determine their relative function during the course of breast cancer progression [3]. Identification of these genes will significantly contribute to the prospect of treatment making choices.

Protease-activated receptor-$_1$ (PAR_1), a G protein-coupled receptor (GPCR), is the first and prototype member of

the mammalian PAR family consisting of four genes. The activation of PAR_1 involves the release of an N-terminal peptide and the exposure of an otherwise hindered ligand, resulting in an exclusive mode of activation. This mode of activation serves as a general paradigm for the entire PAR family [4–6]. While a well-known classical observation points to a close link between hyperactivation of the coagulation system and cancer malignancies, the molecular mechanism that governs procoagulant tumor progression remains poorly defined [7–10]. Thrombin is a main effector of the coagulation cascade. In addition to cleaving fibrinogen, it also activates cells through at least three PARs: PAR_1, PAR_3, and PAR_4. In contrast, PAR_2 is activated by multiple trypsin-like serine proteases including the upstream coagulant proteases VIIa—tissue factor (TF) and Xa, but not by thrombin. It is now becoming well established that human *Par*$_1$, *hPar*$_1$, plays a central role in epithelial malignancies [13, 14, 16]. PAR_2, the second member of the family, is also emerging with central assignments in breast cancer [11, 12]. High levels of *hPar*$_1$ expression are directly correlated with epithelia tumor progression in both clinically obtained biopsy specimens and a wide spectrum of differentially metastatic cell lines [13, 14]. PAR_1 also plays a role in the physiological invasion process of placental cytotrophoblasts during implantation into the uterus deciduas [15]. Trophoblast invasion shares many features with the tumor cell invasion process. It differs, however, by the time-limited *hPar*$_1$ expression, which is confined to the trophoblast-invasive period and is shut off immediately thereafter, when there is no need to invade [13]. This strongly supports the notion that the *hPar*$_1$ gene is part of an invasive gene program. Surprisingly, the zinc-dependent matrix-metalloprotease 1 (MMP-1), a collagenase that efficiently cleaves extra cellular matrix (ECM) and basement membrane components, has been shown to specifically activate PAR_1 [16]. PAR_1-MMP1 axis may thus provide a direct mechanistic link between PAR_1 and tumor metastasis. The mechanism that leads to *hPar*$_1$ gene overexpression in tumor is yet unclear and under current extensive investigation. Although the impaired internalization of PAR_1 that results with persistent signaling and invasion was previously suggested for several breast cancer lines [17], an imbalanced expression between *hPar*$_1$ repressors and activators was proposed, suggesting transcriptional regulation [18]. We found that the mechanism of *hPar*$_1$ overexpression involves enhanced transcriptional activity, whereby enhanced RNA chain elongation takes place in the aggressive cancer cells as compared with the nonaggressive, low metastatic potential cells [19]. Indeed, we have identified the *Egr-1* transcription factor as a critical DNA-binding protein eliciting *hPar*$_1$ expression in prostate cancer cells and the *wt* p53 tumor suppressor as an *hPar*$_1$ transcription repressor [19, 20]. The *wt* form of p53 thus acts as a fine-tuning regulator of *hPar*$_1$ in cancer progression.

2. Prognostic Parameters of PARs

The PARs act as delicate sensors of extra cellular protease gradient to allow the cells to respond to a proteolytically modified environment. The fact that PAR_1 gene and protein overexpression are associated with the aggressiveness of a tumor, *in vivo*, reflect its potential role in cancer dissemination. Furthermore, it assigns PAR_1 as an attractive target for anticancer therapy. On the other hand, the release of an N-terminal peptide during activation and the exposure of an otherwise cryptic internal ligand in PARs endow these receptors with the opportunity to serve as a "*mirror-image*" index reflecting in body fluids the level of PARs on the surface of cancer cells. Hence, PAR_1 and PAR_2 peptides in the blood directly imitate PAR expression serving as a faithful indicator for the extent of cancer progression. While the overexpression of both PAR_1 and PAR_2 takes place on the surface of cancer cells that are being constantly turned over in the body, yet there is no current information as to the half -life of the released peptides. It is envisioned that measuring the level of released peptides may underline the severity of cancer. Another aspect is that the followup levels of PAR_1-released peptides may be instrumental in demonstrating the effectiveness of a given treatment. For example, determining the level of the released PAR_1 and PAR_2, through repeated measurements in the blood stream, may serve as a base line for a patient, and a sensitive indicator for response to a treatment. If the released PAR peptides are becoming gradually low and finally disappear, it may reassure that the tumor is indeed regressing until finally the cancer disappears. In contrast, if the level remains unchanged, it may indicate that the tumor is progressing despite of a given treatment. A critical aspect, however, that needs to be addressed is the prospect of high released $PAR_{1\&2}$ peptides present during inflammation [21, 22]. Therefore, the repeated *followup* of PAR released peptides is necessary for the purpose of demonstrating that during inflammation the high PAR-released peptide level is transient and disappears when the inflammatory response is over. In contrast, in the case of a tumor, the level of PAR-released peptides remains constantly high. The relative contribution of PAR_1 versus PAR_2 during the process of tumor progression is as yet unknown and is under current investigation. One approach to decisively address this issue is by immunohistological staining (of anti-PAR_1 and anti-PAR_2 antibodies, separately) utilizing tissue microarray biopsy specimens on a large pool of primary breast cancer biopsy specimens representing invasive carcinoma. Such analysis will determine the relative percentage of PAR-positive individuals in a given cancer patient pool. Whether PARs join the triple negative population (ER-, PR-, and Her-2/Neu, an indicator of disease aggressiveness)—or perhaps stands independently as a prognostic marker—needs to be evaluated.

3. PARs as Target for Therapy

Importantly, PAR_1 cellular trafficking and signal termination appear to occur in a different mode than other GPCRs. Instead of recycling back to the cell surface after ligand stimulation, activated PAR_1 is sorted to the lysosomes where it is degraded [23, 24]. While cellular trafficking of PAR_1 impinges on the extent and mode of signaling, the identification of individual PAR_1 signaling partners and their contribution to breast cancer progression remain to be elucidated.

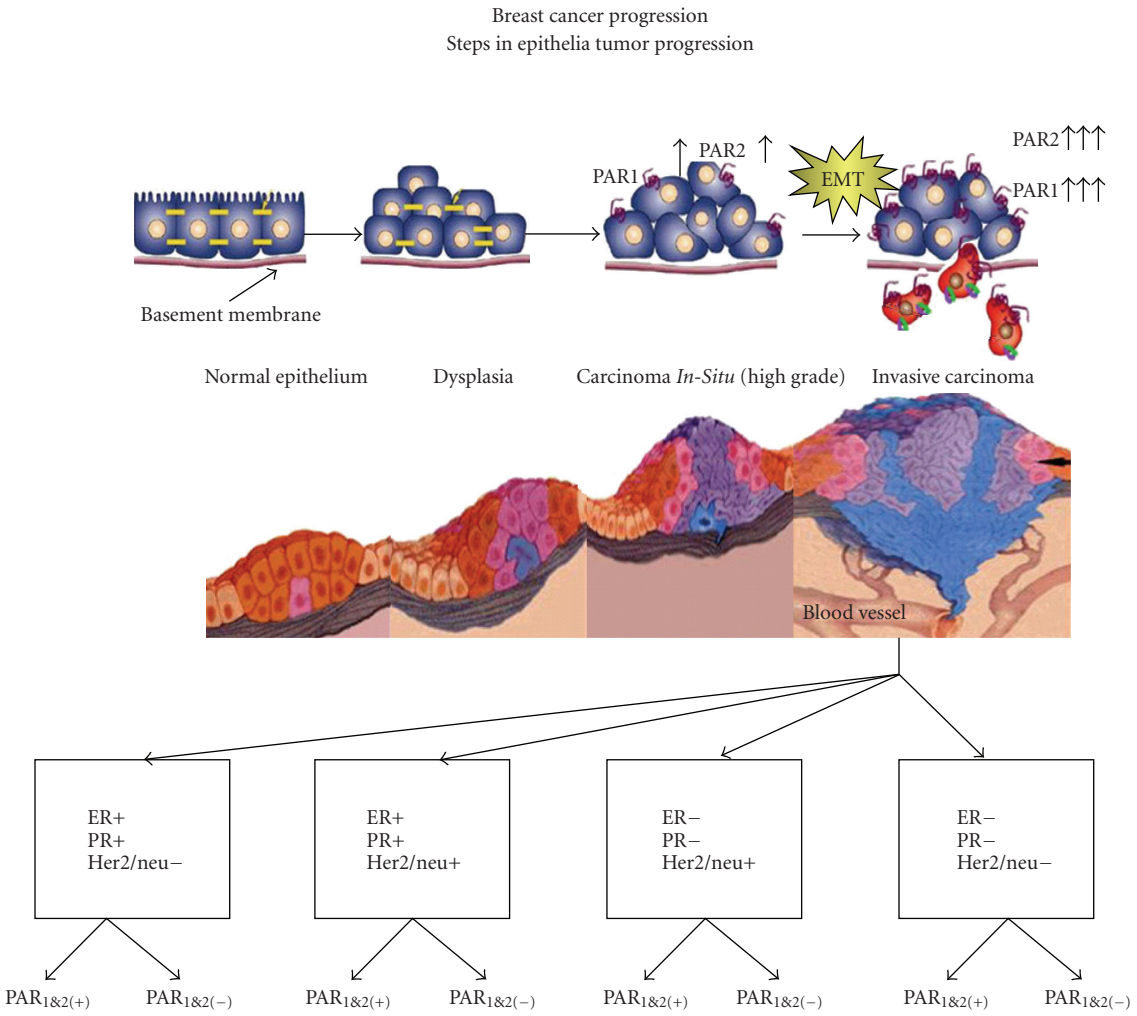

FIGURE 1: Steps in breast cancer progression. Subtypes definition of breast cancer according to ER, PR, and Her2/neu status. Additional categorization is suggested including PARs status.

We have adopted the approach of utilizing a truncated form of $hPar_1$ gene devoid of the entire cytoplasmic tail to demonstrate the significant role of PAR_1 signaling in breast tumor progression. This was demonstrated in a xenograft mice model of mammary gland tumor development, *in vivo* [25]. Along this line of evidence, we have identified PAR_1 C-tail as a scaffold site for the immobilization of signaling partners. In addition to identifying key partners, we have determined the hierarchy of binding and established a region in PAR_1 C-tail critical for breast cancer signaling. This minimal binding domain may provide a potent platform for future therapeutic vehicles in treating breast cancer. The above-described outcome is a brief summary of the detailed experimental approach illustrated bellow.

The functional outcome of MCF7 cells overexpressing various $hPar_1$ constructs *in vivo* was assessed by orthotopic mammary fat pad tumor development. MCF7 cells overexpressing either persistent $hPar_1$ *Y397Z* or *wt* $hPar_1$ constructs (e.g., MCF7/*Y397Z* $hPar_1$; MCF7/*wt* $hPar_1$) markedly enhanced tumor growth *in vivo* following implantation into the mammary glands, whereas MCF7 cells overexpressing truncated $hPar_1$, devoid of the entire cytoplasmic tail, behaved similarly to control MCF7 cells in vector-injected mice, which developed only very small tumors. The tumors obtained with MCF7/*wt* $hPar_1$ and MCF7/*Y397Z* $hPar_1$ were 5 and 5.8 times larger, respectively, than tumors produced by the MCF7/empty vector-transfected cells. Histological examination (H&E staining) showed that while both MCF7/*wt* $hPar_1$ and MCF7/*Y397Z* $hPar_1$ tumors infiltrated into the fat pad tissues of the breast, the MCF7/*Y397Z* $hPar_1$ tumors further infiltrated the abdominal muscle. In contrast, tumors produced by empty vector or truncated $hPar_1$-transfected cells were capsulated, with no obvious cell invasion. Tumor growth can also be attributed to blood vessel formation [26, 27]. The $hPar_1$-induced breast tumor vascularization was assessed by immunostaining with antilectin and anti-CD31 antibodies, showing that both MCF7/*Y397Z hPar* $hPar_1$ and MCF7/*wt* $hPar_1$ tumors were intensely stained. In contrast, only few blood vessels were found in the small tumors of empty vector or truncated $hPar_1$. Thus, both MCF7/*wt*

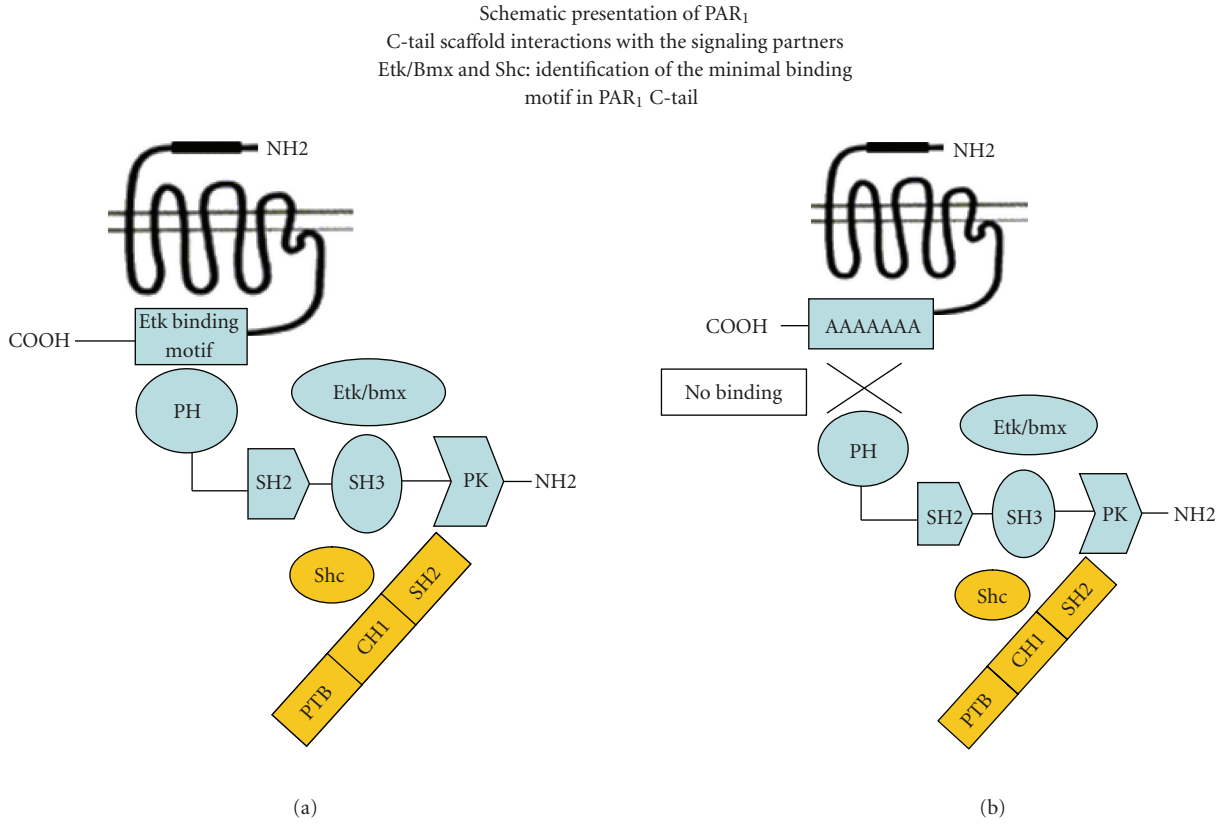

Figure 2: Activation of PAR_1 leads to the association of Etk/Bmx with PAR_1 C-tail. This association is mediated through Etk/Bmx PH-domain enabling next the binding of Shc. The site of the "signal binding" domain (e.g., Etk/Bmx, as a prime signaling partner) in PAR_1 has been identified. Insertion of successive replacement of A residues forming a PAR_1 mutant incapable of binding Etk/Bmx showed impaired capabilities of PAR_1 induced invasion and migration. This site provides therefore a platform for the development of future therapeutic medicaments in breast cancer.

hPar$_1$ and MCF7/*Y397Z hPar*$_1$ cells were shown to effectively induce breast tumor growth, proliferation, and angiogenesis, while the MCF7/truncated *hPar*$_1$ and MCF7/empty vector-expressing cells had no significant effect. This experimental results highlight the significance of PAR_1 signaling in PAR_1-induced breast cancer progression.

4. Antibody Array for Protein-Protein Interactions Reveals Signaling Candidates

Next, in order to identify specific PAR_1 signaling components, the following approach was utilized. To detect the putative mediator(s) linking PAR_1 to potential signaling pathway, we examined a custom-made antibody-array membranes. When aggressive breast carcinoma MDA-MB-435 cells (with high *hPar*$_1$ levels) were incubated with the antibody-array membranes before and after PAR_1 activation (15 minutes), the following results were obtained. Several activation-dependent proteins which interact with PAR_1, including ICAM, c-Yes, Shc, and Etk/Bmx, were identified. Of these proteins, we chose to focus here on Etk/Bmx and Shc.

The epithelial tyrosine kinase (Etk), also known as Bmx, is a nonreceptor tyrosine kinase that is unique by virtue of being able to interact with both tyrosine kinase receptors and GPCRs [28]. This type of interaction is mainly attributed to the pleckstrin homology (PH) which is followed by the Src homology SH3 and SH2 domains and a tyrosine kinase site [29]. Etk/Bmx-PAR_1 interactions were characterized by binding of lysates exhibiting various *hPar*$_1$ forms to GST-PH-Etk/Bmx. While *Y397Z hPar*$_1$and *wt hPar*$_1$ showed specific association with Etk/Bmx, lysates of truncated *hPar*$_1$ or JAR cells (lacking PAR_1) exhibited no binding. A tight association between the PAR_1 C-tail and Etk/Bmx was obtained, independent of whether *wt* or kinase-inactive Etk/Bmx (KQ) was used [29, 30].

5. Hierarchy of Binding

Next, we wished to determine the chain of events mediating the signaling of PAR_1 and the binding of Shc and Etk/Bmx to PAR_1 C-tail. Shc is a well-recognized cell signaling adaptor known to associate with tyrosine-phosphorylated residues. To this end, analysis of MCF7 cells that express little to no *hPar*$_1$ were ectopically forced to overexpress *hPar*$_1$ gene. When coimmunoprecipitation with anti-PAR_1 antibodies following PAR_1 activation was performed, surprisingly, no

Shc was detected in the PAR_1 immunocomplex. Shc association with PAR_1 was fully rescued only when MCF7 cells were initially cotransfected with Etk/Bmx, resulting with abundant assembly of Shc in the immunocomplex. Thus, Etk/Bmx is a critical component that binds first to activated PAR_1 C-tail enabling the binding of Shc. Shc may bind either to phosphorylated Etk/Bmx, via its SH2 domain, or in an unknown manner to the PAR_1 C-tail, provided that Etk/Bmx is present and is PAR_1-bound complex. One cannot, however, exclude the possibility that Bmx binds first to Shc, and only then the complex of Etk/Bmx-Shc binds to PAR_1.

The functional consequences of the Etk/Bmx binding was further evaluated by inserting mutations to the "signal-binding" site. We prepared $hPar_1$ constructs with successive replacement of the designated seven residues (378-384; CQRYVYS) with A, termed as $hPar_1$-7A. This HA tagged mutant, HA-$hPar_1$-7A, completely failed to immunoprecipitate Etk/Bmx. In contrast, in the presence of HA-*wt* $hPar_1$, potent immunoprecipitation was obtained. We thus conclude that the critical region for Etk/Bmx binding to PAR_1 C-tail resides in the vicinity of CQRYVYS. The physiological significance of PAR_1-Etk/Bmx binding is emphasized by the following outcome. Activated MCF7 cells that express $hPar_1$-7A mutant failed to invade Matrigel-coated membranes. In-contrast, a potent invasion was obtained by activated *wt* $hPar_1$. This outcome highlights the fact that by preventing the binding of a key signaling partner to PAR_1 C-tail, efficient inhibition of PAR_1 pro-oncogenic functions, including the loss of epithelial cell polarity, migration, and invasion, is obtained (see Figures 1 and 2 for *wt* and mutated PAR_1 C-tail and the ability to form a scaffold complexes with the signaling partners). Elucidation of the PAR_1 C-tail binding domain may therefore provide a potent platform for future therapeutic vehicles in treating breast cancer.

The same approach may be utilized to identify a prime signaling partner for PAR_2. This will eventually lead to characterization of a minimal PAR_2 C-tail binding region. Generation of peptides that can enter the cells via adding Tat or penetratin, or alternatively, addition of either myristoylation, or another lipid moiety, will assist the peptides to cross the cell membrane. These peptides may prove as effective therapeutic inhibitors of PARs-induced breast cancer growth and development. Along this line of evidence, successful PAR_1-derived peptides termed "pepducin" were developed by the group of Kuliopulos A [31]. This group has demonstrated that PAR_1-induced breast tumor in a mouse model, *in vivo,* is blocked by the cell-penetrating lipopeptide "pepducin," P1pal-7, which is a potent inhibitor of cell viability in breast carcinoma cells expressing PAR_1. It has been shown that P1pal-7 is capable of promoting apoptosis in breast tumor xenografts and significantly inhibits metastasis to the lung.

In summary, PARs may provide a timely effective challenge for developing valuable prognostic vehicles and also critical targets for therapy in breast cancer. While the PAR prognostic vehicles stem from the extracelluar portion of the receptors, we offer the intracellular C-tail site as potential targets for therapy in breast cancer. What is the relative contribution of PAR_1 versus PAR_2 in breast cancer tumor growth and development is yet an open question and a subject of current evaluation.

Conflict of Interests

The authors have declared that no conflict of interests exists.

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Research Article

Short-Term Prognostic Index for Breast Cancer: NPI or Lpi

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Axillary lymph node involvement is an important prognostic factor for breast cancer survival but is confounded by the number of nodes examined. We compare the performance of the log odds prognostic index (Lpi), using a ratio of the positive versus negative lymph nodes, with the Nottingham Prognostic Index (NPI) for short-term breast cancer specific disease free survival. A total of 1818 operable breast cancer patients treated in the University Hospital of Leuven between 2000 and 2005 were included. The performance of the NPI and Lpi were compared on two levels: calibration and discrimination. The latter was evaluated using the concordance index (cindex), the number of patients in the extreme groups, and difference in event rates between these. The NPI had a significant higher cindex, but a significant lower percentage of patients in the extreme risk groups. After updating both indices, no significant differences between NPI and Lpi were noted.

1. Introduction

In women with an operable breast cancer, the lymph node status is amongst the most important prognostic parameters for disease free survival (DFS). An operable breast cancer patient was defined as "any consecutive patient with an invasive breast cancer without a contraindication for primary surgery". This excludes patients with metastatic disease and those previously treated for breast cancer such as those receiving neoadjuvant systemic treatment. A positive axillary lymph node status is associated with a clear increase in risk of recurrence and mortality. Moreover, patients with at least four positive lymph nodes have a worse prognosis compared with those with three or less positive nodes [1]. Examination of axillary lymph nodes for tumor involvement can be performed by axillary lymph node dissection or sentinel lymph node biopsy [2]. Sentinel lymph node biopsy enables an accurate nodal staging by examination of one or a few sentinel lymph nodes, while obviating invasive surgery of axillary dissection [3]. In case the sentinel lymph node is positive, a complete axillary lymph node dissection is performed [4]. However, guidelines defining the minimum number of lymph nodes to examine in axillary lymph node dissection and/or sentinel lymph node biopsy are not available. As the likelihood of finding positive nodes in the axilla increases with the number of nodes removed during axillary lymph node dissection an increasing number of studies is examining the prognostic value of the nodal ratio, which were nicely reviewed by Woodward and coworkers [5].

Furthermore, the established Nottingham Prognostic Index (NPI) [6] is put under debate as it takes only the number of positive lymph nodes into consideration. Vinh-Hung and colleagues introduced a ratio-based nodal prognostic index based on the empirical log odds of nodal involvement [7, 8]. Similar to the NPI, this log odds prognostic index (Lpi) is computed based on the tumor size, histological grade, and axillary nodal involvement. The latter considering the number of negative nodes besides the

number of positive lymph nodes. Using the Surveillance, Epidemiology and End Results (SEER release 2005, n = 7526) public database, they compared the established NPI with the alternative Lpi prognostic index. They reported a better prognostic separation with regard to overall survival as well as breast cancer specific survival between risk groups when defined by the Lpi [7]. This study aimed at evaluating the prognostic value of Lpi in a dataset of 1818 operable breast cancer patients from our institution.

2. Materials and Methods

A total of 2,024 operable female breast cancer patients who were treated in the University Hospitals of Leuven between 2000 and 2005 and underwent lymph node dissection were available for this study. Only patients without prior pathologic sentinel lymph node evaluation (n = 1,838) were included. After exclusion of 20 patients lacking information on size, grade, or lymph node status, 1,818 patients remained for analysis. None of these patients received neoadjuvant therapy. Tumor characteristics and lymph node status were retrieved from pathology reports and together with clinical data gathered in our central breast cancer database. The lymph node status was defined according to AJCC criteria. Isolated tumor cells were considered as lymph node negative. Surgical treatment consisted in wide local excision plus axillary dissection followed by whole breast radiotherapy plus a boost on the tumour bed, or modified radical mastectomy when breast-conserving surgery was not indicated. Chest wall RT following mastectomy was given to patients with T3 or T4 tumors, with positive lymph nodes or with positive tumour section margins. Irradiation to the internal mammary chain was performed only in cases of axillary lymph node involvement or medial tumour sites. Endocrine therapy (HT) was prescribed if the expression ER and/or PR were present. Although tamoxifen 20 mg/day for five years was the standard HT, many postmenopausal women also received an oral aromatase-inhibitor for a period of five years. Anthracycline-based chemotherapy (CT) was given if patients were classified as intermediate or high risk for relapse but endocrine sensitivity, NPI, and age at diagnosis were also important when deciding upon systemic adjuvant therapy.

Patients were followed until June 2009 for cancer recurrence through clinical records or through contact with the general practitioner by phone. Median follow-up time was 5.92 year (interquartile range (IQR): 4.33–7.12 year). During the follow-up period, 67 patients developed local recurrence and 193 developed distant metastasis. For patients with bilateral cancer at first diagnosis, the worse NPI and concordant Lpi value were taken into account.

NPI and Lpi were calculated using tumor size, histological grade, number of positive and/or negative lymph nodes. Tumor size was defined as the maximum diameter of the tumor in cm. Histological grading of tumors was performed according to the Ellis and Elston system [9]. The NPI was computed as 0,2xtumor size (cm) + grade(1–3) + nodal score. The nodal score was defined (1) when no nodal involvement was present, (2) when ≤3 positive lymph nodes, and (3) when >3 positive lymph nodes were present. The Lpi was computed using an equation published in [8]: size(cm) + 1 if grade > 2(0 otherwise) + log((npos + 0.5)/(nneg + 0.5)), where npos, en, and nneg are the number of positive and negative nodes, respectively.

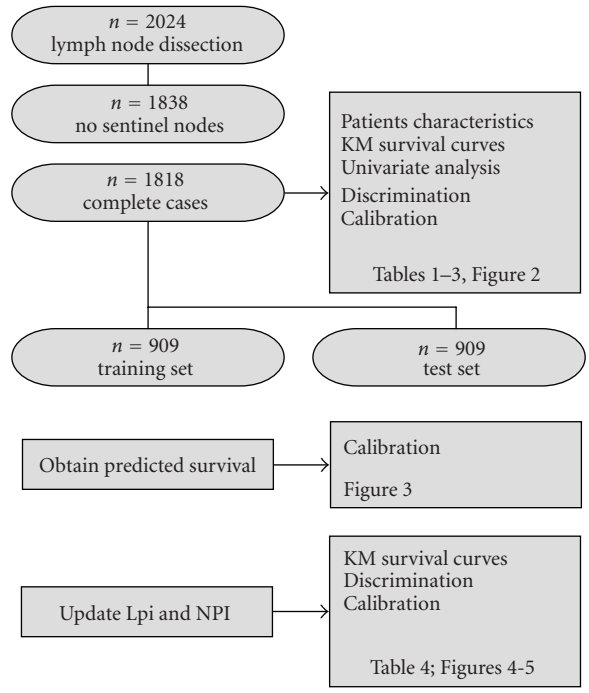

Figure 1: Flow chart: from the 2024 patients treated in our institution, 1,818 were eligible for this study. This cohort was used to summarize patient characteristics, Kaplan-Meier survival curves, and univariable survival analysis. The discriminating ability of the NPI and Lpi were compared on this cohort. Calibration of both models was checked on the test set (a random half of the data), after obtaining the predicted survival curves on the training set. The same training set was used to update both models. Kaplan-Meier curves, discrimination, and calibration ability of the updated models were calculated on the test set.

3. Statistical Analyses

Patients were followed from the date of surgery until a breast cancer-related event (locoregional recurrence or distant metastasis) occurred. In case no event was observed within the study time, patients were censored at the last date of followup. This time will be denoted as the disease-free survival time (DFS). Survival curves are calculated by means of the Kaplan-Meier (KM) method. The logrank test is used to test for statistical significant differences in survival. All variables used in both prognostic models were used in a univariate Cox model to check their relevance on our dataset. The prognostic models are validated on two levels: discrimination and calibration. The discrimination ability is summarized in the concordance index (cindex) [10]. For clinical practice, a model categorizing patients in the most extreme risk groups is preferred [11]. Therefore,

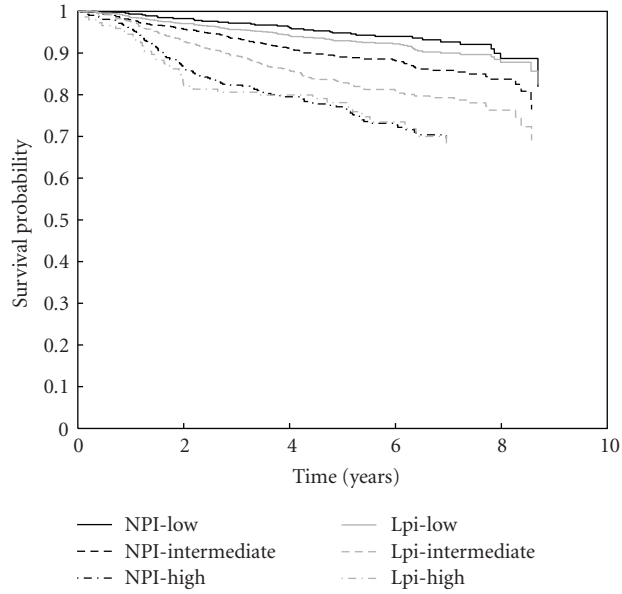

Figure 2: Kaplan-Meier survival curves for risk groups according to NPI (black lines) and Lpi (grey lines). The solid, dashed, and dot-dashed lines represent the low, intermediate, and high-risk group, respectively. The survival curves for both low-risk groups and for both high risk groups do not differ significantly. However, since the Lpi classifies significantly more patients into the low-risk group, the survival for Lpi intermediate risk patients is significantly lower than for NPI intermediate patients.

the percentage of patients in the low and high risk groups are calculated (EXT%). Additionally, the difference in event rates between high and low risk groups are reported (EvR). The event rate is calculated as the number of events divided by the total followup of all patients in the group. The inverse of the event rate is interpreted as the number of years one has to wait before an event is expected to occur. Bootstrap adjusted 95% confidence limits were calculated on all validation measures, using 1000 bootstrap samples of the dataset. Calibration plots are made to check whether the predicted survival chance corresponds with the true survival. Therefore, the test data were divided into five groups according to the value of the NPI (Lpi). For each group, the observed survival as calculated by the Kaplan-Meier estimator at 5 years and the median predicted survival at 5 years are calculated. The calibration plot summarizes the results. However, since the NPI was developed on a dataset containing 351 operable breast cancer patients from 1976–1981 and the Lpi on the SEER 2005 dataset containing 7526 patients from 1988–2004, it is reasonable to assume that these patient populations differ from ours. Not only is the treatment continuously changing, but the number of examined lymph nodes may differ from centre to center. Therefore, our data was randomly divided in a training and test set (both containing 50% of the data). The model predicted survival was obtained from the training set. Calibration was then checked on the test set.

Steyerberg [12] proposed model updating in cases where it is expected that patient populations between training and test sets will differ. Populations can differ due to temporal or spatial differences. To overcome these transportability problems, the NPI and Lpi were updated on half of our dataset (training set, see above) and validated on the remaining part (test set). The models were updated by model revision [12], which involves a re-estimation of the coefficients. As in [6], the updated prognostic indices are built from the resulting Cox proportional hazard regression model.

To categorize patients into risk groups, 1000 bootstraps of the training data set were used. In each bootstrap the lower and higher cutoff values were varied in steps of 0, 2. The pair leading to the largest cindex on most of the bootstraps was selected. Figure 1 gives a flow chart of the data and analysis flow. All statistical analyses were carried out using the software packages SAS 9.1.3 service pack 4, the level of significance being set at $\alpha = 0,05$.

4. Results

4.1. Patient Characteristics. The median age at diagnosis was 57 years (IQR 48-67). All patients were treated with local surgery. The median number of dissected lymph nodes for all patients was 16 (IQR 12–21). A total of 38% (695/1818) of patients had lymph node metastasis with on average 4.11 (IQR 1–5) involved nodes. The median follow-up time was 5.92 year (IQR 4.33–7.12 year). Patients' characteristics are summarized in Table 1.

4.2. Discrimination and Calibration. Figure 2 illustrates the KM curves for NPI and Lpi risk groups. The number of patients in the low, intermediate, and high risk group for the NPI are 464, 934, 420, and for the Lpi 934, 736, 148, respectively. Although the Lpi has much more low risk patients than the NPI, DFS of both groups does not differ significantly ($P = .2176$). Due to the allocation of patients to lower risk groups in the Lpi the DFS of the intermediate Lpi group is significantly lower than that for intermediate NPI patients ($P < .001$). As a first analysis, the significance of all variables in both the NPI and Lpi is checked in a univariate Cox model (Table 2). All variables included in both prognostic indices remain significant in our series. Table 3 summarizes the performance of both models. The NPI has a significantly higher cindex, but a significantly lower EXT%. The models do not differ in the difference between high and low risk event rates. Figure 3 shows that the NPI is better calibrated than the Lpi.

4.3. Model Updating. The major difference between the NPI and the Lpi lies in the substitution of staging of the number of positive lymph nodes by the ratio of positive versus negative nodes. In [13, 14], it was indicated that the ratio of positive versus the total number of examined lymph nodes is a better prognostic than the number of positive nodes per se. However, the number of examined nodes can differ between centres, which would result in different model coefficients. Therefore, both models are updated [12] using a random half of the dataset (training set). The updated

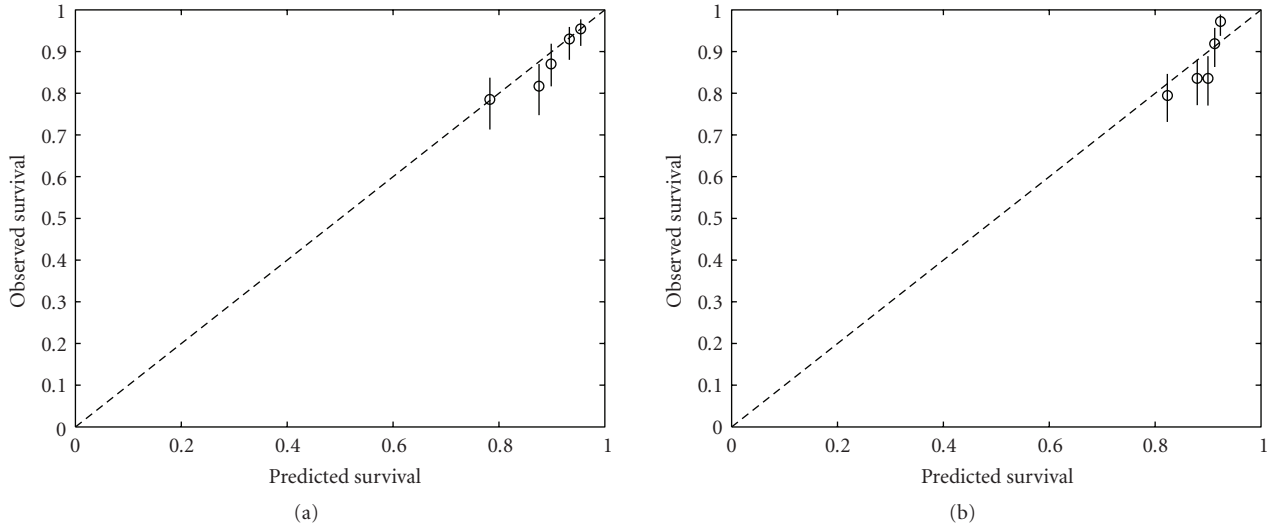

Figure 3: Calibration plot: (a) NPI, (b) Lpi. The test data are divided into 5 groups according to the value of the NPI (Lpi). For each group, the observed survival as calculated by the Kaplan-Meier estimator at 5 years and the median predicted survival at 5 years are plotted (circles). Ideally, the circles should lie on the dashed line. 95% conference intervals on the observed survival probabilities are represented by the vertical lines. The NPI is better calibrated than the Lpi.

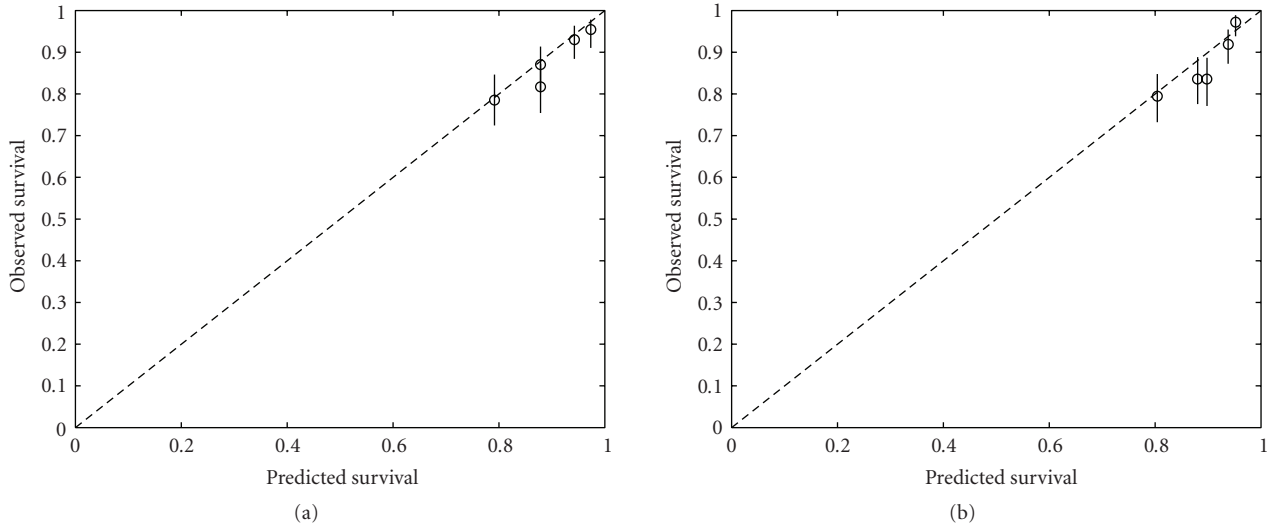

Figure 4: Calibration plot after updating the models: (a) NPI, (b) Lpi. The test data are divided into 5 groups according to the value of the NPI (Lpi). For each group, the observed survival as calculated by the Kaplan-Meier estimator at 5 years and the median predicted survival at 5 years are plotted (circles). Ideally, the circles should lie on the dashed line. 95% conference intervals on the observed survival probabilities are represented by the vertical lines. Both indices are well calibrated.

NPI is built as 0.8xgrade + 0.4xnodal score, where grade and nodal score are defined as previously. The updated Lpi is built as 0.9 (if the grade is larger than 2, zero otherwise) + 0.4xlog((npos + 0.5)/(nneg + 0.5)). The size was dropped from both formulas since the effect was no longer significant when taken together with the nodal score or lymph node ratio. Table 4 summarizes the performance of both updated models. No statistical differences are noted between both models. Figure 4 illustrates that both updated models are well calibrated.

Using 1000 bootstrap samples of the training set, the optimal cutoffs for NPI and Lpi were obtained as {2.85, 3.65} and {−0.90, 0.00}, respectively. Figure 5 illustrates the KM survival estimates for the updated risk groups. Survival curves for corresponding NPI and Lpi groups did not differ significantly ($P > .25$). Comparing the original models with the updated models the concordance for the NPI was 0.93, whereas the concordance for the Lpi was 0.77. Since the updated NPI corresponds that well with the original NPI, the major difference is due to the change of the threshold

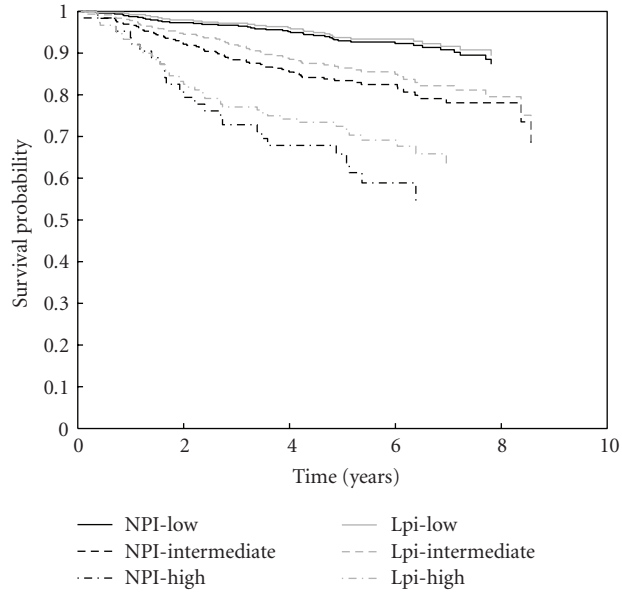

Figure 5: Kaplan-Meier survival curves for risks groups according to the NPI (black lines) and Lpi (grey lines). The solid, dashed, and dot-dashed lines represent the low, intermediate, and high risk group, respectively. The survival curves for corresponding NPI and Lpi groups do not differ significantly.

values. For the Lpi, both the updating of the coefficients and the change in threshold leads to a better model.

5. Discussion

In our institution, the NPI is taken into account to estimate a patient's risk for relapse of breast cancer and to decide upon the modality of systemic therapy. The combined expression of ER, PR, and HER-2 is also taken into account, not only for prognostic but also for predictive purposes. This paper investigated the potential additional benefit of the number of involved lymph nodes or the lymph node ratio.

Various prognostic factors have been described in breast cancer of which the axillary lymph node status and NPI are considered to be the most important ones. However, a tendency towards assessment of the nodal ratio can be seen with various studies indicating a better prognostic stratification of patients by nodal ratio than by number of positive lymph nodes [13–21]. Furthermore, an alternative nodal prognostic index for the established NPI has been put forward [8]. In the present study, we retrospectively reviewed all clinical charts and pathological reports from 1818 operable breast cancer patients which were treated in our institution between 2000 and 2005. We determined the NPI and Lpi value of all patients, updated NPI, and Lpi, categorized patients into risk groups and evaluated the results with regard to disease-free survival.

In contrast to the report of Vinh-Hung and colleagues, we did not find a significant improvement of the Lpi above the NPI, although we previously confirmed the superiority of the LNR above the number of positive lymph nodes [14].

Table 1: Patients' characteristics.

Characteristic	No. of Patients (n = 1818)	%
Age, years		
Median	57	
Lower-upper quartiles	48–67	
Histologic grade		
low	242	13.31
intermediate	823	45.27
high	753	41.42
Tumor size, cm		
Median	2.3	
Lower-upper quartiles	1.5–3.5	
No. of lymph nodes removed		
Median	16	
Lower-upper quartiles	12–21	
No. of positive lymph nodes		
Median	0	
Lower-upper quartiles	0-1	
Range	0–42	
Pathologic nodal stage (pN)		
0	1123	61.77
1–3	456	25.08
> 3	239	13.15
Node ratio		
Median	0.04	
Lower-upper quartiles	0.03–0.14	
Adjuvant treatment		
No adjuvant treatment	37	0.02
Radiotherapy	1519	83.55
Chemotherapy	655	36.03
Endocrine Therapy	1430	78.66

After updating both models, this conclusion remained. Since no significant differences between the Lpi and the NPI are noted, the NPI is preferred above the Lpi. The former index is less dependent on centre since it only includes well-defined variables. The Lpi on the contrary includes the number of examined lymph nodes which might vary according to the centre/surgeon. Therefore, Lpi would need updating to acquire the same performance as the NPI.

We believe this study to be of great value despite its smaller size in comparison with the study of [8]. First, our patient population is representative for the overall breast cancer population with respect to the median age at diagnosis and number of patients in the various NPI risk groups [22, 23]. Second, all patients in the study were diagnosed and treated in a single institution by one multidisciplinary team. However, our study also has its weaknesses such as the lack of overall survival analyses and a relatively short follow-up time.

Table 2: Cox univariate analysis of prognostic variables included in the NPI and Lpi, and NPI and Lpi.

Variable	HR	95% CI	*P*-value
Size (cm)	1.081	1.028–1.136	.0023
pN			
low versus high	0.527	0.453–0.612	<.0001
pN			
intermediate versus high	0.630	0.512–0.775	<.0001
Grade			
low versus high	0.432	0.350–0.533	<.0001
Grade			
intermediate versus high	0.642	0.559–0.737	<.0001
Lymph node ratio	1.427	1.328–1.533	<.0001
Grade			
low and intermediate versus high	2.644	2.057–3.398	<.0001

HR: hazard ratio
pN: pathological nodal status

Table 3: Measures of model performance of NPI and Lpi. Significant better performance is indicated in bold.

measure	NPI	Lpi	95% CI for the difference between NPI and Lpi
cindex	**0.69**	0.66	0.01; 0.04
EvR	0.04	0.04	−0.01; 0.02
EXT%	48.6	**59.5**	−0.14; −0.08

EvR: difference in event rate in high versus low risk patients
EXT%: percentage of patients classified into the most extreme risk groups
CI: confidence interval.

Table 4: Measures of model performance of NPI and Lpi, after updating. Significant better performance is indicated in bold.

measure	NPI	Lpi	95% CI for the difference between NPI and Lpi
cindex	0.69	0.69	−0.02; 0.02
EvR	0.08	0.05	−0.00; 0.05
EXT%	0.66	0.67	−0.04; 0.01

Difference in event rate in high versus low risk patients
EXT%: percentage of patients classified into the most extreme risk groups
CI: confidence interval.

6. Conclusion

This study aimed at evaluating the prognostic value of the Lpi with respect to disease-free survival. However, besides the Lpi various other prognostic indices are under investigation as improvement of the established NPI [22, 24]. Based on the results from our study, we can conclude that the Lpi does not perform better than the NPI.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

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Review Article

Issues Related to Sentinel Lymph Node Assessment in the Management of Breast Cancer—What Are Relevant in Pathology Reports?

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Most cancer centers now perform sentinel node (SN) biopsies. The limited number of SNs sampled compared with an axillary dissection has allowed more comprehensive lymph node analysis resulting in increased detection of micrometastases. Many node-negative cases are now reclassified as micrometastatic. Recent research on SN biopsy focuses on whether axillary dissection is always necessary when the SN is positive. Some subgroups of patients have a higher risk of more nodal metastases when completion axillary dissections were performed. This paper summarizes the different studies and examines what are the clinically relevant items to report on SN node pathology: volume or size of nodal metastasis, location within the node, extranodal extension, number of involved SN(s) and non-SN(s), total number of SN, and total number of nodes on axillary dissection, if performed.

1. Introduction

Breast cancer is estimated to have an incidence of 22,700 and will cause 5400 cancer deaths among females in Canada in 2009 as per Canadian Cancer Society Statistics [1]. Better prognostic factors to aid oncologists in making treatment decision will benefit a significant number of patients. The nodal staging of breast cancer generally involved a level I and II axillary dissection. To reduce the risk of surgical complications, sentinel node (SN) biopsy has been widely used in the last decade.

Nodal ratio (absolute number of involved nodes/number of nodes resected) was recently proposed to have a greater prognostic value than absolute number of involved nodes [2–7]. Since the paper by Woodward et al. [8] from the International Nodal Ratio (NR) Working Group, there are a few more studies confirming this [9]. In a recent study, relapse free and overall survival rates were not different according to the absolute number of involved nodes ($P = .166$, $P = .248$, resp.) [10], but on multivariate analysis, the NR was an independent prognostic factor for relapse free and overall survival (Hazard ratio, HR = 4.246, $P < .001$; HR = 7.764, $P < .001$), respectively.

Different dividing lines for NR have been used in the literature. Our previous work showed a survival benefit for regional nodal radiotherapy (RT) when the NR of axillary nodes is 0.25 or more [11]. In this study, patients were categorized into three NR groups; low (LNR, ≤25%), medium (MNR, >25% to ≤75%) and high (HNR, >75%) nodal involvement. This categorization follows previous literature using British Columbia data [2] and American data [8, 12]. Truong et al. found that 25% is a good dividing line for grouping [2].

With *sentinel node (SN) mapping* technique, the minimum number of nodes required for accurate staging becomes less. This is because SN biopsy technique uses radioisotope and dye to guide the search for first drainage node(s) accurately. SN biopsy correctly identifies the involved node which could be missed by axillary node dissection without any guidance [13]. Analysis of frozen section of SNs is an accurate method for metastasis detection, allowing axillary dissection when positive at the same operative setting [14].

Controversies of sentinel node assessment abound. This paper aims to *summarize and analyze* the current management of breast cancer following SN biopsy. Recommendations to target readers (clinical oncologists and pathologists) are suggested.

2. Material and Methods

A search of PubMed and the proceedings of the American Society for Therapeutic Radiology and Oncology (ASTRO) and American Society of Clinical Oncology (ASCO) annual meeting books was performed and selected relevant articles and abstracts pertinent to SN assessment and prognostic relevance.

3. Results and Discussion

The limited number of SNs compared with an axillary dissection has allowed more comprehensive lymph node analysis resulting in increased detection of micrometastases. Many women previously classified as node-negative are now reclassified as having micrometastatic nodal involvement. As a result, our nodal classification and cancer staging have evolved to recognize the continuum of nodal tumor burden rather than a simplistic dichotomous stratification [15]. The pathologist is expected to mount, stain, and microscopically examine serial sections of the SN using hematoxylin and eosin (H&E) staining. Despite recommendations from the College of American Pathologists and the American Society of Clinical Oncology, heterogeneity in the approach to SN evaluation exists. What is needed is adherence to a standardized evaluation protocol. The most important aspect of the SN examination is careful attention to slicing the SN with thickness no more than 2.0 mm and correct embedding of the slices to assure all macrometastases larger than 2.0 mm are identified.

3.1. Is Minimal Lymph Node Involvement Clinically Relevant?

There is an ongoing debate concerning the clinical implications of micrometastases in the SN. Many observational studies have been published but results do not justify conclusions. Bulte et al. [18] of Netherlands looked at the subgroup of patients with micrometastases ($n = 38$) : 3 (7.9%) patients developed distant recurrence. In the group with a tumour-free sentinel node ($n = 503$), 17 (3.4%) distant recurrence and 3 (0.6%) combined regional and distant recurrence were observed. The rates of distant recurrence between the node-negative and micrometastatic cases are not significantly different (Chi-square test, $P = .128$). However, the authors reported that the result may be limited by small sample size. Despite the lack of statistical significance of outcome of pN1mi in reference 18, to an individual patient the worse outcome is still *clinically important*.

Indeed other studies show that the prognosis of patients with pN1mi is significantly worse compared to node-negative patients, in terms of regional and distant recurrence rates [19]. The worse prognosis was further confirmed by a large SEER database study [20]: breast cancer specific survival (BCSS) and overall survival with pNmi disease progressively declined with increasing number of positive nodes and increasing NR.

Table 1: Definition of minimal pathologic lymph node involvement in American Joint Committee on Cancer (AJCC) Staging Manual, seventh edition (2010) [16, 17].

pN0(i−)	No regional lymph node metastases histologically, negative immunohistochemistry (IHC)
pN0(i+)	Malignant cells in regional lymph node(s) no greater than 0.2 mm (detected by H&E or IHC including isolated tumor cell clusters (ITC))
pN0(mol−)	No regional lymph node metastases histologically, negative molecular findings (RT-PCR, reverse transcriptase/polymerase chain reaction)
pN0(mol+)	Positive molecular findings (RT-PCR), but no regional lymph node metastases detected by histology or IHC
pN1	Micrometastases; or metastases in 1–3 axillary lymph nodes; and/or in internal mammary nodes with metastases detected by sentinel lymph node biopsy but not clinically detected
pN1mi	Micrometastases (greater than 0.2 mm and/or more than 200 cells, but none greater than 2.0 mm)

In the MIRROR (Micrometastases and ITCs: Relevant and Robust or Rubbish?) study, almost all participating pathology laboratories used a protocol in which the SN was serially sectioned at least every 150 μm and at a minimum of three levels, with the use of keratin immunohistochemical (IHC) staining if the H&E staining was negative. In contrast, the nonSNs were macroscopically sectioned every 2 to 5 mm, and one section per slice was stained with H&E. The aim was to evaluate the relationship, if any, between ITCs or micrometastases in the regional lymph nodes and clinical outcome in patients who had undergone an SN procedure and who did or did not receive systemic adjuvant therapy. *They found that adjuvant treatment helped to lower the risk of disease events* [21]. The median followup was 5.1 years. This agrees with large studies that included women who received a diagnosis before the SN era; micrometastases, defined as 2 mm or smaller in diameter and including ITCs, were associated with reduced overall survival [22–25]. In these studies, however, the axillary nodes were examined by means of H&E staining at just one level. Thus, we cannot compare these studies with the MIRROR study, which involved a detailed examination of the SN. The few previous studies of SNs were limited by small samples, lack of multivariate analyses, or short followup [26–28].

It is noteworthy that for patients with minimal nodal involvement, the disease-free survival (DFS) was *initially similar but started to fall after the third year compared to node-negative results* [29]. Patients with pN1a and pN1mi/pN0i+, when compared with patients with pN0 disease, were more often prescribed anthracycline-containing chemotherapy

(39.1% versus 33.2% versus 6.1%, resp., $P < .0001$) and were less likely to receive endocrine therapy alone (9.8% versus 19.4% versus 41.9%, resp., $P < .0001$). On multivariate analysis, a statistically significant difference in DFS and in the risk of distant metastases was observed for patients with pN1a versus pN0 disease (HR = 2.04; 95% CI, 1.46 to 2.86; $P < .0001$ for DFS; HR = 2.32; 95% CI, 1.42 to 3.80; $P = .0007$ for distant metastases) and for *patients with pN1mi/pN0i+ versus pN0 disease* (HR = 1.58; 95% CI, 1.01 to 2.47; $P = .047$ for DFS; HR = 1.94; 95% CI, 1.04 to 3.64; $P = .037$ for distant metastases).

In summary, Table 2 shows that pN1mi patients consistently have an HR for events of 1.5 versus node-negative patients. Hence despite smaller studies with shorter followup showing no significant difference in outcome [18, 30], with the best available evidence at the present time, the authors of this paper felt that pN1mi patients tend to have worse outcome than node-negative patients.

In our institute, medical oncologists tend to treat patients with micrometastases (0.2–2 mm node) with adjuvant systemic treatment, while the treatment for nodal metastases <0.2 mm is still debatable. For patients with nodal metastasis ≤2 mm including ITC, the use of nodal radiotherapy is controversial. A muticenters trial for these patients with enough followup duration, and to stratify tumor size, grade and nodal ratio may provide further insight to the role of nodal radiotherapy.

3.2. Completion Axillary Dissection after a Positive SN Biopsy. Another area of recent research on SN biopsy focuses on whether axillary lymph node dissection (ALND) is always necessary when the SN is positive [31]—what is the probability of further nodal metastases in the axilla? Here we examine the available research studies on this issue.

A study of 159 stage T1 or T2 breast cancer patients speculated that axillary dissection *can be avoided in those patients diagnosed of micrometastasis* in the SN [32]. Completion ALND was performed when micro or macrometastases were found in the SN. A total of 40 patients (25%) showed infiltration of the SN. This infiltration was only by micrometastasis in 17 of them (10.7%). Of these 17 patients, only 2 *(11.8%)* showed macrometastasis in the lymphadenectomy specimens.

3.2.1. Which Patients Can Be Safely Selected to Forgo Completion ALND?

Table 2 shows that if micrometastasis is found in a SN, omission of additional ALND may be envisaged by Houvenaeghel et al. with minimal risk for pT1a and pT1b tumors, and pT1a-b-c tumors corresponding to *tubular, colloidal, or medullary* cancers [33].

A study from São Paulo of 1,000 successive patients with SN biopsy from 1998 to 2008 put this issue into context [34]. The mean age was 57.6 years and mean tumor size was 1.85 cm. A total of 72.2% SN were negative and 27.8% were positive, but in 61.9% of the cases, the SN was the only positive one, with 78.4% having macrometastases, 17.3% micrometastases and 4.3% ITCs. After 54 months of followup, there were no recurrences in patients with ITCs, but one local recurrence and two systemic recurrences were observed in the micrometastases group, as well as four local and 30 distant metastases in the macrometastases group. *Among the clinical parameters studied, only tumor size was correlated with metastatic involvement in axillary lymph nodes.* The size of the metastases and the number of positive SN also directly increased the possibility of systemic recurrence.

Volume of disease in the SN is a significant predictor of additional nodal metastasis. In a Memorial Sloan-Kettering Cancer Center study of 505 patients, 251 pN0(i+) and 254 pN1mi: 12% of pN0(i+) and *20*% pN1mi had additional nonSN disease [35]. On multivariate analyses including eight variables, only *lymphovascular invasion* (odds ratio > 2.2, $P < .01$) and *volume of nodal metastasis* as assessed by any method of measurement (method of detection, AJCC, and cell count) were significantly correlated with additional nonSN disease ($P = .04$, $.03$, and $.02$, resp.).

More pathologic risk factors were investigated in another study of 128 patients who had a positive SN biopsy in 2005–2007 [36]. The metastases in each SN were assessed according to their location within the node (subcapsular, mixed subcapsular and parenchymal, parenchymal, multifocal or extensive) and metastatic infiltration of perinodal tissue was also reported. The strong predictors of the axillary lymph node metastasis included the SN metastasis diameter (7.6 versus 4.4 mm) and size classified according to WHO classification (ITC 0 versus 100%, micrometastasis 23.5 versus 76.5%, macrometastasis 51.9 versus 48.1%). The *SN metastases with a diameter of above 3 mm* were associated with approximately twice more frequent ALN metastases. If there is extensive SN metastasis, the highest percentage of ALN metastases was found (65 versus 35%). The weak predictors of ALN metastases were: primary tumor diameter (>2 cm), immunohistochemical HER2 positive status, infiltration of sentinel perinodal tissue by metastasis, histological primary tumour grade.

Two other important concepts to select patients for completion ALND to mention are nomograms and nodal ratio. *Nomograms* or other scoring systems have been used to predict the chance of involvement of nonSNs after a single involved SN is found [37, 38]. The *nodal ratio* concept has been extended to SN biopsy. More than one positive SN and a ratio of positive SN(s) to total SN(s) of greater than 0.5 were found to be predictors for additional axillary nodal involvement in both univariate and multivariate analyses [39]. The number of positive SNs and the SN nodal ratio is an indication of total tumor burden in the SNs and may be a reflection of the propensity of the tumor for further lymphatic invasion in the axillary basin.

3.2.2. What Is the Significance of IHC Positivity in SN Which Is H&E Negative?

The surgeons at the St Vincent's University Hospital in Dublin, Ireland performed SN mapping for breast cancer

Table 2: Important studies on micrometastatic nodes in breast cancer.

Author	Study	Median FU	Patient number	Conclusion
Bulte et al. [18]	7 hospitals in Netherlands	3.8 years	503 pN0 38 pNmi	Local relapse—5(1.0%) versus 1(2.6%) Regional relapse—0% versus 0% Distant relapse—17(3.4%) versus 3(7.9%) Combined locoregional relapse—1(0.2%) versus 0% Combined regional and distant relapse—3(0.6%) versus 0% (n.s., see text for details)
Hainsworth et al. [19]	St Vincent's Hospital, Australia	6.6 years	31/343 occult node metastases found on IHC, plus 10 found on H&E	Among the 31 patients, presence of occult metastases in 2 or more nodes was associated with decreased DFS and OS ($P < .05$)
Truong et al. [20]	Surveillance Epidemiology and End Results database	7.3 years	62,551 pT1–2pN0-: 57,980 pN0, 1818 pNmi, 2753 pNmac >2 mm but <2 cm	10-year BCSS (82.3% versus 91.9%) and OS (68.1% versus 75.7%) in pNmi compared to pN0. (s.s.)
Colleoni et al. [29]	Italian medical oncology department	4.2 years	1959 pT1-3, pN0, pN1mi or pN0i+), or pN1a (single positive node) and M0	pN1mi/pN0i+ versus pN0 disease: HR = 1.58; 95% CI, 1.01 to 2.47; $P = .047$ for DFS; HR = 1.94; 95% CI, 1.04 to 3.64; $P = .037$ for distant metastases.
de Boer et al. [21]	Dutch cohort study of all centers in Netherlands (MIRROR study)	5.1 years	(a) 856 Nmi/ITC without adjuvant therapy, (b) 995 Nmi/ITC with adjuvant therapy, (c) 856 node-negative	Disease events: (a) for Nmi: HR 1.56 (95% CI:1.15-2.13; for ITC:HR 1.50 (95% CI:1.15-1.94) (b) HR 0.57 (95% CI:0.45-0.73)
Houvenaeghel et al. [33]	A French center	—	SN involvement in 388 times (55.4%) by H&E, 312 times by IHC	May omit additional ALND for pT1a and pT1b tumors, and pT1a-c tumors corresponding to tubular, colloidal or medullary cancers

ALND: axilllary lymph node dissection; DFS: disease-free survival; FU: followup; n.s.: statistically nonsignificant; OS: overall survival; SNB: sentinel node biopsy; s.s.: statistically nonsignificant.

from January 1st, 2000 to December 31st, 2006 [40]. All SNs were assessed by serial H&E and IHC sections. Patients with micrometastases (0.2–2 mm) underwent completion ALND. Patients with ITC (<0.2 mm) were individually discussed and an ALND was performed selectively based on additional clinicopathological criteria and patient preference. Patients were followed for a median of 27 months (range 12–72 months). 1076 patients who underwent SN were included for analysis. The experience is unique as it demonstrates the breakdown of cases into each category: 211 (20%) had a positive SN biopsy using H&E. *Forty-nine patients (5%) had a negative SN on H&E which was positive on IHC.* Of these, *15 had micrometastases and underwent an ALND. Two had further axillary nodal disease.* ITC were found in the remaining 34 patients. Sixteen of these patients underwent an ALND. Five of this group had further nodal disease. Therefore, micrometastases and ITCs, detected only by IHC analysis of SNs, are associated with further positive nodes in the axilla in *up to 15*% of patients. However, more research is needed and IHC is not yet the standard procedure in most pathology departments.

3.3. Effect on Survival of Completion ALND. Completion ALND remains the gold standard for patients with a tumor-involved SN. ALND achieves regional control, but its effect on *survival* remains controversial. The *American College of Surgeons Oncology Group (*ACOSOG) Z0011 study randomized clinically node-negative patients who underwent SN biopsy and had 1 or 2 SN with metastases detected by H&E to ALND or no further axillary surgery [41]. Ineligibility criteria were SN metastasis detected by IHC, 3 or more SN positive, third field RT for nodal RT or accelerated partial breast irradiation (APBI). Both groups have tangent breast RT plus *systemic therapy* (which can be hormone or chemotherapy). The 446 patients with SN biopsy alone and 445 patients with SN biopsy plus ALND were similar

Table 3: Summary of results of American College of Surgeons Oncology Group (ACOSOG) Z0011 study.

	ALND	SN biopsy	*P* values
5-year in-breast recurrence	3.7%	2.1%	.16
5-year nodal recurrence	0.6%	13%	.44
5-year overall survival	91.9%	92.5%	.24
5-year disease-free survival	82.2%	83.8%	.13

Table 4: Summary of important aspects of a standard pathology report—this would enable oncologists to make individual decision on management, including completion ALND and adjuvant systemic treatment.

Primary tumor
Multifocal or multicentric
In-situ component
Grade
Necrosis
Lymphovascular invasion
Margin
Histologic subtype
Immunohistochemical HER2, ER, PR
Involvement of nipple or skeletal muscle
Abnormalities of surrounding breast tissue
NODE
Volume or size of nodal metastasis
Location within the node
Extranodal extension
Number of involved sentinel node
Number of involved nonsentinel node
Total number of sentinel node
Total number of nodes on axillary dissection, if performed

in prognostic factors. Median followup is 6.2 years. Table 4 summarizes the results.

So despite the widely held belief that ALND improves survival, no significant difference was recognized by this study of SN node-positive women. Although the study closed early because of low accrual/event rate, it is the largest phase III study of ALND for node-positive women, and it demonstrates no trend toward clinical benefit of ALND for patients with *limited* nodal disease *and given adjuvant systemic therapy*.

Based on the above evidence, the authors of this paper believe that when the estimated risk of nonSN involvement is low enough, a completion ALND is not necessary. Even if there is further involved nonSN(s), they may be treated by systemic therapy or tangent field RT which covers level I and some level II axillary nodes. The survival benefit from radiation is best explained by the prevention of an isolated loco-regional recurrence, which could serve as a source of fatal distant metastases and parallels the difference in the total incidence of distant metastases [42].

3.4. Important Aspects of a Standard Pathology Report. See Table 4.

4. Conclusion

We have summarized the studies and analyzed them as a whole to draw the following conclusions. Misleading studies due to small patient numbers and short followup have clouded the issue of poor outcome of pN1mi before. The authors of this paper felt that pN1mi patients have worse outcome than node-negative patients. Patients with micrometastases or ITC benefit from adjuvant systemic treatment as evident from de Boer et al. [21].

When the *estimated* risk of nonSN involvement is low enough, a completion ALND is not necessary. Even if there is further involved nonSN(s), they may be treated by systemic therapy or tangent field RT which covers level I and some level II axillary nodes. In the ACOSOG study, for clinically node-negative patients who underwent SN biopsy and had 1 or 2 SN with metastases detected by H&E, completion ALND would not affect local recurrence, OS or DFS [41]. Hence provided patients had *limited nodal disease and receive* adjuvant systemic treatment, completion ALND after SN biopsy is not warranted.

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Review Article

Sentinel Node Biopsy for Breast Cancer Patients: Issues for Discussion and Our Practice

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Sentinel node biopsy has been established for several years now as a standard procedure of breast cancer surgery, but there are several variations of the indications and the technique used. This paper provides information regarding several issues of debate for its application as are the selection criteria, the application to patients with multifocal/multicentric breast cancer or DCIS, postneoadjuvant chemotherapy, the necessary number of nodes to be biopsied, the need for lymphoscintigraphy, the technique for frozen section, the factors that may predict nonsentinel nodes (NSNs) involvement, the value of micrometastasis and isolated tumour cells, the internal mammary chain sentinel nodes, and finally the axillary recurrence after SLNB. Our view for these issues is included together with our experience of 430 SLNBs.

1. Introduction

Lymph node status is a key factor in determining the stage of breast cancer and the most appropriate therapy and for predicting the outcome of patients. Accurate identification of sentinel lymph nodes (SLNs) preoperatively is of clinical importance. The results of the NSABP B-32 study indicate the superiority of the SLNB compared to the ALND treatment approach relative to postsurgical morbidity outcomes over a 3-year follow-up period [1]. Also the use of ipsilateral upper arm is not restricted if only SLNB is applied. In the sentinel lymph node (SLN) era, axillary lymph node dissection (ALND) for uninvolved axillary lymph nodes should be considered unnecessary and inappropriate. The sentinel lymph node biopsy sensitivity is more than 90%, its specificity 100%, its accuracy more than 95%, and the axillary recurrence rate is less than 1%. There are still though some disputable issues also on this subject.

2. Selection Criteria for a SLN Biopsy

ASCO Expert Panel published in 2005 guidelines in which SLNB was not acceptable for T3 or T4 tumors, inflammatory breast cancer, ductal carcinoma in situ (DCIS) without mastectomy, nodes suspicious for metastasis, pregnancy, prior axillary surgery, prior nononcologic breast surgery, and after preoperative systemic therapy [2]. Is it possible though to predict to which patients we should not perform the procedure? According to Lynch et al. [3] there are *clinical and pathologic features which are associated with a positive SLN,* such as a palpable tumor, increasing tumor size, increasing histologic grade, and angiolymphatic invasion. Some of the above features are unreliable and for other we do not have information always.

Regarding *tumor size*, SLNB may be acceptable also for patients with T3 or T4b tumors according to Takei et al. [4], even though SLN identification is lower. Yet SLN involvement is higher compared with T1 or T2 tumors, and systemic adjuvant therapy is obviously needed for patients with T3 or T4b tumors. SLNB is only a bridge to further axillary treatment such as ALND or axillary RT for those patients. *Clinical examination of the axilla* is always the first approach to select the candidate for SLNB. This approach though, is inaccurate in 41% of cases, false positive in 53% of patients with moderately suspicious nodes and 23% of patients with highly suspicious nodes. False positive results

are less frequent with larger tumor size and higher histological grade, but are not associated with age, body mass index, or previous surgical biopsy [5]. Nodes clinically suspicious for metastasis should not be considered a contraindication to SLNB, since palpable axillary lymph nodes can be identified and removed by SLNB [4]. It has been recently shown that by applying the procedure also to patients with clinically suspicious nodes, after neoadjuvant treatment, large tumors >2 cm, multifocal disease, and previous excisional biopsy the number of unnecessary ALNDs has been decreased from 26% to 9% [6].

One would consider that there is not a discrete borderline for the selection criteria and this may be anywhere between to perform SLNB to "all patients" and to perform it to "only small tumors with ultrasound guided FNA negative axillary nodes".

In our unit candidates for SLNB are all patients with tumor diameter of less than 3 cm, and with negative axilla both clinically and ultrasonographically. With these selection criteria we have performed 430 SLNBs. The size of less than 3 cm criterion is not supported by the literature, on the contrary. Being very conservative, we have only recently moved to apply SLNB in all T2 tumours.

3. Multifocal (MF) or Multicentric (MC) Breast Cancer

In these cases there is an issue when we consider the diameter of the tumor in relation to the possibility of lymph node metastasis. Tresserra et al. [7] found that lymph node metastasis is related only to the diameter of the largest tumor. Ferrari et al. showed that in 93.3% of patients with multifocal or multicentric cancer the lymphatic pathways from two different sites of injection converged into one major trunk leading to the same SLN(s) and in 6.7%, mainly multicentric cancer, two different pathways found each of them leading to a different SLN [8]. The false negative rate was 7.1% in this study and the authors suggested that both MF and MC tumors do not represent a contraindication for SLNB. On the contrary a French prospective multi-institutional study found that the false negative rate of SLNB for multiple unilateral synchronous breast cancer was 13.6% which is unacceptably high even for small tumors [9]. Regarding MC patients it is generally recommended not to perform SLNB. Multifocality and multicentricity consist a contraindication for SLNB in our unit because we aim for a false negative rate of less than 5.9%. This was achieved during the testing period of the first 100 procedures. Multifocal tumours recently are treated differently, as we perform SLNB for the largest focus on a trial's base.

4. Avoiding SLNB

The most reliable so far method to detect involved axillary nodes is *ultrasound and FNA* of the suspicious on palpation and/or ultrasound nodes. In the study of Deurloo et al. this procedure can find 31% of all tumor-positive axillae (macro + micrometastases) and 41% of all axillae containing macrometastases with maximum cortex thickness being the main feature to predict metastatic involvement [10]. Markedly hypoechoic, thick, or lobulated cortex and eccentric or absence of fatty hillum are the malignant features according to Koelliker et al. [11]. Jung et al. found the sensitivity, specificity, and positive and negative predictive values of the ultrasound alone of axillary LNs for metastatic breast cancer were 54, 91, 75, and 81%, retrospectively. For the US-FNAC, the respective values were 80, 98, 97, and 84% [12]. AUS with needle biopsy reduces the need for SLNB by 54% and affects treatment in patients with cT2 or greater breast cancer [13]. The absence of fatty hillum has the highest positive predictive value of 93% in the study of Garcia-Ortega et al. [14]. From the same study the sensitivity and specificity of axillary ultrasonography are 63.2% and 88.7%, respectively. The sensitivity and specificity of *axillary core biopsy* are 69.1% and 100%, respectively. With this procedure sentinel lymph node biopsy can be avoided in 33% of initial candidates and immediate breast reconstruction was undertaken in 35.1% of the patients with mastectomy and negative axillary core biopsy. Moreover breast cancer is frequently characterized by increased 2-fluoro-2-deoxy-D-glucose uptake, and many studies have shown encouraging results in detecting axillary lymph node metastases. The sensitivity of *FDG-PET scan for detection of axillary lymph node metastases* in the study of Veronesi et al. [15] was low (37%); however, specificity and positive predictive values were acceptable (96% and 88%, resp.). The high specificity of PET imaging indicates that patients who have a PET-positive axilla should have an ALND rather than an SNB for axillary staging. In contrast, FDG-PET showed poor sensitivity in the detection of axillary metastases, confirming the need for SNB in cases where PET is negative in the axilla.

Axillary ultrasound has a sensitivity of around 50% and a specificity of more than 90% in our hands and therefore we rely only on the positive results.

5. Neoadjuvant Chemotherapy

So far SLNB is not acceptable for patients with positive nodes in the axilla at initial diagnosis even if their axillary metastases are downstaged to negative by neoadjuvant chemotherapy. In theory, lymphatic mapping may not accurately show whether nodal metastases exist after preoperative chemotherapy because of excessive fibrosis of the tumour lymphatics and/or the potential obstruction of lymphatic channels with cellular material or tumour emboli [16, 17]. Thus, it is important that the feasibility and reliability of SLNB is determined in this patient group.

Three meta-analyses have been reported that examined the results of SLNB in patients with breast cancer who did not receive preoperative chemotherapy [18–20]. The overall sentinel lymph node identification rate (IR) calculated in the largest of these, determined from data on 28 studies, was 90 per cent. The estimated IR for SLNB after preoperative chemotherapy in the meta-analysis of Xing et al. [21] in which data from the 21 studies were analyzed was 91 per cent. The fact that the rates of sentinel lymph node identification

were similar in pooled analyses suggests that the concerns mentioned previously [16, 17] are not serious. The estimated sensitivity of SLNB after preoperative chemotherapy was 88 (95 per cent, credible interval 84 to 91) per cent, with a false-negative rate of 12 per cent. The overall false negative rates for SLNB determined in the three separate meta-analyses of patients who did not receive preoperative chemotherapy were 8.4 per cent [22], 5.1 per cent [23], and 9 per cent [18]. It is still controversial whether SLNB is acceptable for patients with clinically positive nodes at initial diagnosis who are treated with neoadjuvant chemotherapy, whereas SLNB alone is acceptable for patients with an initial diagnosis of clinically negative axilla who are treated with neoadjuvant chemotherapy. SLN identification rate was 65% in patients with clinically positive nodes at initial diagnosis; however, it was 100% in patients with clinically negative presentation who were treated with neoadjuvant chemotherapy at M. D. Anderson Cancer Center [24]. On the other hand proponents of SLNB would argue that in women who have had preoperative chemotherapy the clinical impact of understaging is less significant, given that they were assigned a clinical stage before chemotherapy and so the decision to give systemic therapy had already been made. Thus SLNB after chemotherapy provides information about residual nodal disease and guides regional therapy. There is also the consideration of NAC downstaging the axilla, converting N1-N2 lymph node status to N0 and also avoiding full axillary dissection in these patients, provided that the false negative rate is low as it was found to be 4.3% in the study of Schwartz et al. [25].

Shimazu et al. [26] proved that intraoperative frozen section (FS) analysis of SLNs is as accurate for neoadjuvant chemotherapy-(NAC-)treated as for non-NAC-treated patients, which indicates that FS analysis of SLNs is a clinically acceptable method for those receiving NAC. The application of SLNB- to NAC- treated patients has been proved to have similar sensitivity, specificity, and accuracy to non-NAC-treated patients (74, 100, and 88%, versus 71, 99, and 90%). The sensitivity of FS analysis for macrometastases is lower for NAC-treated patients (76%) than for non-NAC-treated patients (91%), while that for micrometastases and isolated tumor cells is higher for NAC-treated patients (67%) than for non-NAC-treated patients (31%). However, neither of these differences is statistically significant.

Neoadjuvant chemotherapy can be given not only to patients with locally advanced breast cancer, but also to those with axillary lymph node metastasis and an operable tumor for down-staging and to downsize a tumor in order to perform conservative surgery. However, SLNB after NAC results in a lower identification rate and a higher FNR than SLNB before treatment. Recently, a hybrid imaging device has been developed, which consists of single photon emission computed tomography (CT, SPECT) and a low-dose CT installed on the same platform. This imaging system offers an easy and safe method of performing SLNB under local anesthesia. To identify the initial cancer stage in patients who will be treated by systemic therapy before surgery, SLNB should be performed prior to systemic treatments, according to Iwase et al. [27] by using a well-developed navigating tool, such as SPECT/CT or the radioguided.

Neoadjuvant chemotherapy or hormone therapy for operable cancer is still under investigation in our unit and axillary clearance is a standard procedure for these patients.

6. DCIS

DCIS is pathologically diagnosed only after complete removal of the tumor, and the incidence of accompanying microinvasion increases when the tumor is palpable and large, is of high grade, or if the patient is young [28]. The ASCO guidelines showed a cutoff diameter of 5 mm or larger, for which SLNB is recommended for patients with an initial diagnosis of DCIS [2]. In addition, SLNB is recommended for patients who will undergo mastectomy for the treatment of DCIS, because the ability to perform SLNB is lost after removal of the breast.

Our standard procedure is to be prepared and perform SLNB (radioguided or with blue dye) when frozen section results show DCIS. We prefer to exhaust our efforts not to need a second operation, which is necessary in almost 15% of cases where an infiltration or microinfiltration is found on final histology.

7. Lymphoscintigraphy

The need to perform lymphoscintigraphy prior to SLNB is another issue. It has been proved beneficial in showing that at least 1 radioactive SLN will be identified intraoperatively, but it does not accurately predict the number of SLN in 40–50% of the patients [29, 30]. The number of hot spots in preoperative mapping should serve as a rough indicator of the smallest number of nodes the surgeon should attempt to resect, but not the exact number of nodes expected to be found.

Since we do not do internal mammary chain SLNB, we find lymphoscintigraphy not helpful from the surgical point of view except in cases with a history of prior sentinel node, axillary dissection, and plastic surgery. Nevertheless our nuclear medicine department finds its images reassuring for the efficacy of their job.

8. Number of Sentinel Nodes

The improvement of experience with the blue dye procedure along with the addition of radioisotope marking of the SLN contributed to the increase of the number of SLN are biopsied. Palpable tumors, surgeon's inexperience, and dermal injection are associated with greater than 4 SLNs identified. All 3 of these factors remain significant on multivariate analysis [31]. Low and littlejohn [32] suggest that the optimal number of SLN to harvest, after intradermal injection of both isotope and blue dye, is two. In their study 33 patients had positive SLN results. If only the first SLN was analyzed, 87.9% of those positive biopsies would have been discovered. Two SLNs raised the predictive value to 97.0%. Lynch et al. [3] identified a mean number of 2.86 (range, 1–8)

SLNs after periareolar injection of radiolabeled technetium sulfur colloid on the day of surgery. Among the 38 patients with a positive SLN (30.2%), the hottest node was the first positive SLN in 27 patients (71.1%). The first positive SLN was the first node removed in 31 patients (81.6%) and the second node in 37 patients (97.4%); it was removed in all patients by the third SLN. These data support the trend of limiting SLN biopsy to 3 lymph nodes. Removing all SLNs with radioactive counts greater than 10% of the ex vivo counts of the hottest SLN does not increase accuracy. The false negative rates were 14.3% and 4.3% for patients with a single sentinel node versus multiple sentinel nodes removed, respectively, in the study of Wong et al. [33]. The blue dye injection alone was the only factor independently associated with identification of a single SLN and patient age, tumor size, tumor location, surgeon's previous experience, and type of operation were not significant.

It is also our finding that blue dye staining only leads in most cases to a single SLNB. Our average SLN number is 1.9 by following the rule of 10%.

9. Factors That Predict Nonsentinel Nodes (NSNs) Involvement

It is accepted in the community that a positive SLN frozen section should be followed by ALND. Attempts have been made to identify factors that predict non sentinel nodes (NSNs) involvement. The rate of NSNs involvement increases proportionately to the size of both SN metastases and primary tumor, while no significant correlation was found for lymphovascular invasion. At univariate and multivariate analysis of findings from cases with multiple probe-detected hot nodes, positivity in more than one hot node is the strongest predictor of NSN involvement [34]. More than one positive SLN and a ratio of positive SLNs to total SLNs of greater than 0.5 were found by Tan et al. [35] to be predictors for additional axillary nodal involvement in both univariate and multivariate analyses. The number of positive SLNs and the ratio of positive SLNs to total SLNs is an indication of total tumor burden in the sentinel nodes and may be a reflection of the propensity of the tumor for further lymphatic invasion in the axillary basin. Another assumption made is that some patients may benefit from a more conservative surgical approach to their axillae, perhaps limited to sentinel node biopsy only or to axillary procedures restricted to the group of axillary nodes in close proximity to those designated as sentinel nodes. This assumption was made when Samoilova et al. [36] found that all patients with sentinel node tumor deposits < or =5 mm had three or fewer positive nodes; 95% were sentinel node-positive only, and 91% had single-node involvement. Nine models have been developed until now to predict non SN status in patients with SN metastasis. Four models are nomograms: the Memorial Sloan-Kettering Cancer Center nomogram (MSKCC nomogram), the Mayo nomogram, the Cambridge nomogram, and the Stanford nomogram. Three models are scoring systems: the Tenon score, the score from the M.D. Anderson Cancer Center (MDA score), and the score of the group of Saidi. Finally, two are recursive partitioning tools developed by the group of Kohrt. Those models have been compared by Coutent et al. [37]. They found that all models do not perform equally, especially for the subgroup of patients with only micrometastasis or ITC in the SN overall, the MSKCC nomogram and Tenon score outperform other methods for all patients, including the subgroup of patients with only SN micrometastases or ITC, but need extensive testing before they are put into clinical practice. A new perspective of non SLN metastasis prediction is the presence of extracapsular invasion of the SLN and it was studied by Fujii et al. [38]. It seems to be a strong predictor of residual disease in the axilla. All cases of positive nodes in NSLN in these series had extracapsular invasion at the metastatic SLNs. Furthermore, the absence of ECI of SLN was significantly associated with the absence of metastasis in the NSLN ($P < .001$).

Our experience with 12.5% chance of other non-SLN infiltrated on ALND when one only SLN is infiltrated does not allow us not to proceed to ALND in these circumstances. The case of only one SLN with microinfiltration is still under investigation because of the small number of patients.

10. Intraoperative Assessment

The need for intraoperative assessment of the SLN is not under discussion any more as it saves the patient from a second operation most of the time. False negative rate of frozen section is found to be 5–25% percent (not surprisingly greater for micrometastases) and a second operation cannot be avoided always. Its sensitivity may be improved by multilevel sectioning of the lymph node and by histochemistry [39–41].

Imprint cytology has been tried and is still practiced, but failed to achieve results similar to frozen section. The meta-analysis of 31 studies published in 2005 showed that pooled sensitivity of imprint cytology was 63% and specificity was 99%. Pooled sensitivity for macrometastases was 81% and that for micrometastases 22%. Frozen sectioning had better sensitivity than imprint cytology in three of four direct comparisons [42]. More recent studies comparing frozen section and rapid immunohistochemistry to touch imprint cytology did not change these findings [43].

Ultrarapid cytokeratin IHC assay is a procedure that does not exceed 20 min. Compared to *frozen hematoxylin-eosin (H&E) stain* has a sensitivity of 85% versus 70%, a specificity of 100% for both and accuracy rate of 96% versus 92%, respectively [44]. Ultrarapid IHC may detect also sentinel node micrometastasis and isolated tumor cells (ITCs) [44–46]. Serial sections with a spacing of 150 microns between following sections seems to increase the ability of IHC to detect ITCs [46]. *One Step Nucleic Amplification* which is a method that amplifies cytokeratin 19 mRNA and measures its amount which is directly related to the size of metastatic foci. This is a procedure that is completed in 30 min. In a multicentric study in Japan it was found that its concordance rate to the histochemical investigation is 98.2% the specificity is 96.5% [47]. Concerns are raised though for the inability to determine the actual size of nodal metastases which is important for therapeutic decisions and the inability to

determine the true false positive and negative rate, since the tissue has been used for RNA isolation.

Frozen section with ultra rapid cytokeratin IHC is the way we proceed. In our series SLN was found negative on H&E and positive on IHC in 3.17% of patients and the discrepancy between H&E and IHC was significantly less common when more than one SLN were examined (1.6% versus 3.7%, $P < .01$).

11. Micrometastasis and Isolated Tumor Cells

The importance of detecting micrometastasis (MM) (0.2 mm –2 mm) and isolated tumor cells (ITCs) (<0.2 mm) in an ALN is unknown. Should its detection in a SLN on ultra rapid IHC lead to ALND? In the study of Dabbs et al. [48] 13.6% of the patients that were IHC positive had ALN macrometastasis in a solitary ALN. Of the patients with micrometastatic SLNs 8.1% had a solitary positive ALN, 6.1% of which were macrometastases. Overall 9.0% with traditionally defined SLN micrometastases of 2.0 mm or less had a solitary ALN macrometastasis. There was a significant difference in the means of SLN tumor sizes for the SLN-positive/ALND-negative (4.5 mm) versus SLN-positive/ALND-positive (10.1 mm) patients. When the 2 recently published interpretations of the TNM definitions were applied to cases of low-volume sentinel lymph node (SLN) involvement and their corresponding non-SLNs for reclassification as micrometastasis or ITC, the rates of non-SLN metastases associated with SLN ITCs were 8.5% and 13.5%, respectively [49]. The prognostic impact of micrometastases and ITCs is still under investigation. Isolated tumour cells or micrometastases in regional lymph nodes were associated with a reduced 5-year rate of disease-free survival among women with favourable early-stage breast cancer who did not receive adjuvant therapy in the study of de Boer et al. In patients with isolated tumour cells or micrometastases who received adjuvant therapy, disease-free survival was improved [50]. Ten-year breast cancer-specific survival (BCSS) and overall survival (OS) in pNmic breast cancer were found by Truong et al. to be significantly lower compared to pN0 disease (BCSS 82.3% versus 91.9%, $P < .001$ and OS 68.1% versus 75.7%, $P < .001$) [51]. Park et al. showed that ITC have no impact on survival at a median 8.2 years of followup, whereas MM shows a trend toward poorer disease-free survival (DFS) ($P = .091$, log rank) and distant disease free survival (DDFS) ($P = .066$) and significantly reduced BCSS ($P = .016$). In multivariate analyses, detection of MM is an independent prognostic factor for DDFS ($P = .025$) and BCSS ($P = .01$) in adjuvant untreated patients. The evidence so far shows that micrometastases in axillary lymph nodes have prognostic impact. This is not found for ITC. Those findings support the use of systemic adjuvant therapy in patients with MM [52]. Axillary recurrence could also be a threat for those patients, and it was found that one patient with an SLN micrometastasis (1 of 33; 3%) and 1 patient with an SLN macrometastasis (1 of 14; 7%) developed an axillary recurrence with distant metastasis at 84 months and 28 months, respectively [53]. The group from MSKCC found that young age, estrogen receptor negative status, high MSKCC nomogram score, and chemotherapy were associated with ALND. The practice of selectively limiting ALND to IHC-only patients thought to be at high risk and to patients for whom the identification of additional positive nodes may change systemic therapy recommendations seems to be a safe and reasonable approach. Among patients who had ALND ($n = 95$), 18% had a positive non-SLN. No axillary recurrences were observed in this series with a median followup of 6.4 years. The percentage of patients who were recurrence-free after 5 years was 97 [54].

There is not though a general agreement for the proper way of sentinel node specimen handling in order to achieve finding all MM and ITCs. There are institutions doubting the necessity of multiple level sectioning [55] and other supporting the Milan proposal of sectioning at 50-micron intervals and for each level, one section stained with hematoxylin and eosin and the other section immunostained for cytokeratins using a rapid immunocytochemical assay, claiming that this way the detection of metastases is increased by 7.8% [56–58]. American College of Pathologists guidelines of 2009 are that the SN should be bivalved along the longitudinal axis, serially sectioned at 1.5- to 2.0-mm thickness blocks; each block should be sectioned at 3 levels and examined using routine H&E stains. They consider controversial the routine use of immunohistochemical (IHC) staining or other molecular approaches.

We perform ALND for all patients with MM. We do not proceed to ALND for patients with ITC if it is the only positive node among 2 or more SLN. If MM is found on regular histology we discuss with the patient the options of doing nothing or having ALND or axillary RT, informing her also of the existing risk of leaving an infiltrated node in her axilla.

12. Internal Mammary Chain Sentinel Nodes

Lymphatic mapping for sentinel lymph node (SLN) biopsy has demonstrated extra-axillary drainage in up to 35% of patients. In the subset of patients with tumours 1 cm or less in size and no ALNM, information on IMN status would provide important information. In these cases, the presence of IMN metastases would change the staging from stage I to stage IIIB, according to the current tumour, node, and metastasis classification. More importantly, it would influence these patients' adjuvant treatment [59]. Peritumoral isotope injection contributes to internal mammary chain (IMC) sentinel lymph nodes visualization in 28.75% of patients according to Bourre et al. [60]. IMC biopsy failed in 4% of patients. IMC sentinel node was infiltrated in 4.8% of biopsies performed. Prophylactic irradiation of the IMC was indicated in 376 patients. Therefore such information should make it possible to personalize treatment for patients with stage cT1 mammary cancer and thereby avoid needless internal mammary radiation therapy in a large number of patients (93.4% in this study). By intratumoral isotope injection and blue dye injection the IMC sentinel node was

visualized in 21.5% of patients and could be harvested in 87% from the study of Estourgie [61]. IMC SLN contained tumor in 17% of those harvested and in 7% IMC nodes were positive whereas the axilla was tumor free. There was a change of management in 29% of the patients with a successful IMC-SLNB, including institution or omission of radiotherapy to the IMC, adjuvant systemic therapy, or omission of the axillary node dissection. In the European Institute of Oncology study IMC nodes were found in 88% of patients, and 8.8% were positive which modified the radiotherapy and systemic treatment [62].

IMC nodes have only lately been a subject of reconsideration in our unit and the same is the site of radiotracer injection as by the subdermal injection we use IMC nodes that are not visualized. For the time being we perform a biopsy from the IMC LNs close to the inner site tumours.

13. Axillary Recurrence after SLNB

The incidence of axillary recurrence after tumor negative sentinel node biopsy**,** in the study of Bulte et al. [63], is 0.6% (3/541). An event occurred in the 11% of patients with a micrometastasis in the sentinel node. This was not significantly different from the patients with a tumour-free sentinel node. In the same study was observed a non-significant different risk of distant disease in case of micrometastases compared to a tumour negative sentinel node. Also the accuracy of SLNB in multicentric/multifocal breast cancer was comparable with that observed in unifocal breast cancer with low false negative rate and no axillary recurrence in the study of Holwitt et al. [64]. Despite a lower rate of SLN positivity in patients undergoing SLNB only, axillary recurrence was not observed and none of the 52 patients experienced axillary recurrence (median followup 4.8 years). In 2008 a meta-analysis was published of 48 selected studies concerning 14959 sentinel node-negative breast cancer patients followed for a median of 34 months [65]. Sixty-seven patients developed an axillary recurrence, resulting in a recurrence rate of 0.3%. The sensitivity of the sentinel node biopsy was 100%. Uni- and multivariable variable analyses showed that the lowest recurrence rates were reported in studies performed in cancer centers, in studies that described the use of (99 m)Tc-sulphur colloid, and also when investigators used the superficial injection technique or evaluated the harvested sentinel nodes with haematoxylin-eosin and immunohistochemistry staining ($P < .01$). These results suggest that the sentinel lymph node procedure is a reliable and accurate instrument for staging of patients with early breast cancer.

Following the criteria we have set our axillary recurrence rate is 0.3% and this happened to one patient who did not complete chemotherapy.

14. Conclusions

Most of the above discussed issues are still in debate**.** Large tumour size and multifocality not contraindication for SLNB if we accept a slightly lower identification and increased false negative rate. Axillary ultrasound with FNA or core biopsy is accepted as helpful, because of its high specificity, in order to decrease the number of SLNBs. The role of SLNB in relation to neoadjuvant chemotherapy is still in debate and the same applies to DCIS. Lymphoscintigraphy is not helpful. Two or three are the optimum number of SLNs to be biopsied. There are no widely accepted rules to predict the non-SLN metastasis. Frozen section with ultra rapid cytokeratin assay is the most preferred procedure for its sensitivity in defining lymph node micrometastasis, which is related to poorer prognosis. Internal mammary chain SLNB may change the management in few patients. The axillary recurrence rate with SLNB is acceptably low and this allows us to try and expand its indications.

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Review Article

Testing for HER2 in Breast Cancer: A Continuing Evolution

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Human epidermal growth factor receptor 2 (HER2) is an important prognostic and predictive factor in breast cancer. HER2 is overexpressed in approximately 15%–20% of invasive breast carcinomas and is associated with earlier recurrence, shortened disease free survival, and poor prognosis. Trastuzumab (Herceptin) a "humanized" monoclonal antibody targets the extracellular domain of HER2 and is widely used in the management of HER2 positive breast cancers. Accurate assessment of HER2 is thus critical in the management of breast cancer. The aim of this paper is to present a comprehensive review of HER2 with reference to its discovery and biology, clinical significance, prognostic value, targeted therapy, current and new testing modalities, and the interpretation guidelines and pitfalls.

1. Introduction and HER2 Biology

In 1981, Shih et al. discovered novel transmissible genes which caused transformation of NIH 3T3 cells upon transfection of DNA obtained from rat neuroblastomas [1]. Subsequently, the same group identified a 185,000 Dalton phosphoprotein obtained from the sera of young mice injected with secondary transfectants containing neuroblastoma transforming sequence [2]. This *neu* oncogene was later identified in genomes of fetal rat neuro/glioblastomas cell lines derived from tumors induced by ethylnitrosurea [3]. The nucleic acid sequence of the neu gene was homologous to the erb-B oncogene and the neu-associated tumor antigen p-185 was antigenically related but distinct from the epidermal growth factor (EGF) receptor. Two other groups by screening the human genomic library using v-erbB as screening probes independently isolated similar erb-B related genes HER2 [4] and c-erbB-2 [5]. Upon further analysis, neu, HER2, and c-erbB-2 were identified as identical genes mapping on the same chromosome location [4, 6]. In 1985, the amplification of this gene in DNA prepared from tissue of human mammary carcinoma was first demonstrated by King et al. [7].

HER2 is a member of the epidermal growth factor (EGF) receptor family which consists of four members: EGFR (HER1, erbB1), HER2 (erbB2), HER3 (erbB3), and HER4 (erbB4). The HER2 gene is located on chromosome 17q12 and encodes a 185-kDa protein product which is a transmembrane receptor protein with tyrosine kinase activity [8–10]. The receptor is structurally composed of an extracellular ligand-binding domain, transmembrane domain, and an intracellular tyrosine kinase catalytic domain. Upon activation by a ligand, the receptors dimerize and undergo transphosphorylation to activate various intracellular signaling pathways which mediate cell proliferation and differentiation [11, 12]. The cellular mechanism of HER2 activation is not completely understood, and there is no known stimulatory ligand for HER2 receptor homodimers. The HER2 receptor can, however, dimerize with other members of the EGFR family to form heterodimers, and these heterodimers involving HER2 have been shown to be more potent and stable [13]. In addition, crystal structures of rat HER2 have revealed a constitutively activated extracellular domain in the absence of a ligand [14].

Soon after its discovery, several in vivo and in vitro studies highlighted the oncogenic potential of HER2. Overexpression of HER2 was shown to be associated with cellular transformation and tumorogenesis in NIH 3T3 cells and human mammary epithelial cells [15–17]. In transgenic

mice, overexpression of HER2 led to development of mammary tumors and induction of metastatic disease [18–20].

2. Prognostic Value of HER2

The prognostic value of HER2 amplification in human breast cancers was first determined by Slamon et al. in 1987 [21]. They evaluated tissues from 189 primary breast cancers and determined the role of HER2 as an independent prognostic factor. HER2 amplification was also shown to be a predictor of overall survival and time to relapse [21]. Currently, there are at least 107 published studies involving 39,730 patients that have discussed the prognostic significance of HER2 gene amplification (as assessed by southern blot, slot blot, polymerase chain reaction [PCR], fluorescent in situ hybridization [FISH] and chromogenic in situ hybridization [CISH]), and protein overexpression (as analyzed by western blot, immunohistochemistry [IHC], and enzyme-linked immunosorbent assay [ELISA]) [22]. Of these, 95 (88%) studies showed HER2 gene amplification or protein overexpression in breast cancer as an important predictive factor by either univariate or multivariate analysis. Multivariate analysis was performed on 93 studies of which 68 (73%) showed HER2 as an independent adverse prognostic factor. However, in 13 (12%) studies there was no correlation between prognosis and HER2 status [22].

In node-positive patients, HER2 amplification or protein overexpression has been shown to be a poor predictor of clinical outcome [21, 23–35]. A recent study by Gilcrease et al. has shown that any degree of HER2 overexpression (1+, 2+ or 3+) was associated with increased tumor recurrence and decreased patient survival in a node-positive cohort of breast cancer patients ($n = 91$) treated with doxorubicin-based chemotherapy without trastuzumab [36]. A different study showed a distinct, intermediate outcome in patients with low-level HER2 amplification by FISH, with a ratio of 1.5–2.2, compared to HER2 unamplified tumors and tumors with HER2 ratios greater than 2.2 [37].

The predictive value of HER2 in node negative patients has been contentious. While some studies verify the adverse predictive value in node negative patients [24, 32, 35, 38–47], others have found no significant correlation with clinical outcome [23, 25, 29–31, 48–51]. The differences in these study conclusions may be attributed to a multitude of factors including differences in the number of patients, patient population including those receiving systemic adjuvant therapy, length of followup, and most importantly HER2 status determination and interpretation techniques.

3. Predictive Value of HER2

In addition to the prognostic significance in breast cancer, HER2 amplification and protein expression has been shown to predict and modulate response of conventional chemotherapeutic agents.

3.1. Combination Chemotherapy. Conflicting studies have been reported regarding the benefit of combination chemotherapy with cyclophosphamide, methotrexate, and fluorouracil (CMF) in HER2-positive tumors. Some studies have shown decreased responsiveness of HER2-positive tumors to CMF therapy. Gusterson et al. reported a randomized study involving 1,506 breast cancer patients enrolled in the international (Ludwig) breast cancer study group trial V [30]. The patients were divided into subgroups of lymph-node positive ($n = 746$) and lymph-node negative ($n = 740$) patients. The patients in the node-positive group were given prolonged chemotherapy or a single cycle of perioperative chemotherapy (PeCT), and patients in the node-negative group were given single cycle of PeCT or no chemotherapy. They concluded that for node-positive patients, the effect of prolonged CMF chemotherapy, and for node-negative patients, the effect of PeCT on disease-free survival, was greater in HER2-negative tumors when compared to HER2-positive tumors defined as focal or diffuse membrane positivity by IHC [30]. Similar results were shown in a subgroup of breast cancer patients ($n = 179$) with low-risk lesions without significant in situ component [43]. In this subgroup, the HER2-positive tumors (focal or diffuse membrane staining by IHC) showed significant decrease in disease-free survival at 5 years (40% versus 80%; $P < .0001$) and overall survival ($P = .0001$) compared to HER2 negative tumors [43].

In contrast to these observations, a controlled clinical trial involving 386 node positive breast cancer patients with a 20-year followup who received 12 monthly cycles of adjuvant CMF ($n = 207$) or no further treatment after radical mastectomy ($n = 179$) showed that both HER2-positive (intermediate or strong membrane staining by IHC) and HER2-negative tumors benefited from treatment which was assessed by relapse-free survival and cause specific survival compared to the untreated patients [52]. These findings were confirmed by other large randomized study which had a median followup of 28.5 years [53].

3.2. Anthracycline-Based Chemotherapy. Though some studies have indicated that patients with locally advanced HER2 overexpressing breast cancers receiving prolonged or high-dose anthracycline-based chemotherapy show no significant change in survival [54], treatment failure [55], and development of distant metastasis [56] when compared to HER2-negative patients, most studies show benefit of anthracycline-based chemotherapy in HER2-positive tumors. Of 1572 patients with lymph node-positive early breast cancer enrolled in Cancer and Leukemia Group B (CALGB) trial randomized to receive high, moderate, and low doses of cyclophosphamide, doxorubicin, and fluorouracil, 442 random tumor samples were obtained and assessed for HER2 expression by IHC [57]. The results indicated that patients with high HER2 expression (≥50%) who received high-dose chemotherapy had a significantly longer disease-free survival and overall survival as compared to the patients with no or low HER2 expression (<50%)

[57]. Similar observations of improved response to high-dose anthracycline-based chemotherapy in HER2 amplified lymph node-positive breast cancers have also been shown by other studies [58, 59].

This was further confirmed in a recent large randomized study involving tissues from 710 premenopausal women with axillary lymph node-positive breast cancer where amplification of HER2 (HER2 to chromosome 17 ratio of ≥ 2) was associated with clinical responsiveness to anthracycline containing chemotherapy containing cyclophosphamide, epirubicin and fluorouracil (CEF) when assessed for relapse-free survival and overall survival compared to patients receiving CMF or tumors that lacked amplification of HER2 [60]. Anthracyclines are topoisomerase inhibitors, and the response to these agents in HER2-positive tumors is postulated to be due to coamplification of topoisomerase II α (topo2a) gene which is located close to the HER2 gene on chromosome 17 [61–63]. Studies have shown that amplification of topo2a occurs exclusively in presence of HER2 amplification and that in the majority of tumors, topo2a amplification correlates with topo2a overexpression [64].

3.3. Tamoxifen. Approximately 75% of all invasive breast carcinomas are positive for estrogen receptors (ER) or progesterone receptors (PR) [65]. Even though HER2-positive tumors show a significantly decreased expression of ER or PR in comparison to HER2-negative tumors, a substantial proportion still express ER or PR [66]. Patients with advanced breast cancer expressing hormone receptors (HR) show increased (70%–80%) response to Tamoxifen therapy, though overall up to 50% of HR-positive tumors will not benefit, and approximately 10% of HR-negative tumors will respond to treatment [67]. Experimental and clinical evidence particularly in advanced-stage cancer have suggested an association between HER2 overexpression and resistance to endocrine therapies in general [68–73]. In a recent prospective study of 516 consecutive stage I-II patients, clinical outcome after 5–10 years following tamoxifen-based adjuvant therapy was compared between HR-positive/HER2-positive subgroup ($n = 51$) and HR-positive/HER2-negative subgroup ($n = 129$) [74]. Cases were considered HER2-positive if membrane staining in >1% was identified in tumor cells. The study concluded that the disease-free survival and overall survival in patients receiving Tamoxifen alone or after chemotherapy was significantly lower in HR+/HER2+ group when compared to HR+/HER2− group [74]. In another retrospective study, node-negative breast cancer patients randomly assigned to 2-year adjuvant Tamoxifen or no further therapy were analyzed for HER2 protein overexpression by IHC [75]. After a median followup of 12 years, univariate analysis showed that adjuvant Tamoxifen significantly prolonged disease-free survival and overall survival in HER2-negative cases whereas it had no effect in HER2-positive cases (membrane staining in >10% cells) [75].

In contrast to the above, a randomized controlled trial of 282 patients with ER positive tumors treated with adjuvant oophorectomy and Tamoxifen were evaluated for HER2 protein expression [76]. Univariate analysis showed risk reduction for all treated patients in both HER2-positive ($n = 73$) and HER2-negative subgroups ($n = 209$) with a greater benefit in the HER2-positive group [76]. In another study by Berry et al., HER2 status in 651 ER-positive, node-positive patients was evaluated by three different methods (IHC, FISH, and PCR), and clinical outcome was evaluated after Tamoxifen therapy [77]. They concluded that the disease-free survival and overall survival in the patients receiving Tamoxifen was not influenced by the HER2 status of the tumors [77].

3.4. Taxanes. Paclitaxol (Taxol), one the first taxanes examined in clinical trials has been shown to be effective against many cancers considered refractory to conventional chemotherapy. Paclitaxol exerts its cytotoxic effect by inhibiting microtubule disassembly and promoting tubulin polymerization, thus disrupting cell division [78]. Though in vitro studies have demonstrated resistance to taxanes in transfected mammary cells overexpressing HER2 [79, 80], in vivo studies have shown contradictory results. Baselga et al. studied the sensitivity of taxanes in women with metastatic breast cancer [81]. The response rate for taxanes was significantly greater in HER2-positive tumors (65%) versus HER2-negative tumors (36%). This sensitivity remained even after controlling for confounding variables which correlated with HER2 overexpression [81]. Similar benefits from paclitaxel containing regimens have also been shown by other studies in patients with HER2 gene amplification or protein overexpression and metastatic breast cancer [82, 83]. Contrasting to these observations, a randomized study involving 474 women showed that the response rate and overall survival were not related to HER2 status, and there was a trend towards shorter median time to treatment failure among women with HER2-positive tumors [84].

4. HER2-Targeted Therapy

4.1. Discovery of Trastuzumab. The high incidence of HER2 gene amplification and protein expression in breast cancer and its prognostic and predictive value make HER2 an attractive target for development of therapeutic agents. In 1985, soon after the discovery of HER2, a monoclonal anti-p185 antibody was shown to revert neu-transformed NIH 3T3 cells into a nontransformed phenotype [85]. Monoclonal antibodies targeting the extracellular domain of HER2 were subsequently developed by several laboratories [86–88]. Several other in vitro studies have confirmed the antineoplastic properties of monoclonal antibodies directed against HER2 expressing tumor cells demonstrated by inhibition of anchorage-dependent growth [89, 90], monolayer tumor growth [91], and colonies in soft agar [91–93] or by sensitizing the HER2 overexpressing cells to tumor necrosis factor alpha [92]. In addition, in vivo studies of monoclonal antibodies directed against HER2 have also shown to inhibit tumor cell growth in transgenic mice [90, 93].

The use of these murine antibodies, however, is limited clinically due to the development of neutralizing human antibodies upon long-term use. To circumvent this dilemma, one of the most potent growth inhibitory anti-p185HER2, designated muMAb4D5 was humanized by gene conversion mutagenesis [91, 94]. This fusion gene (rhuMAb HER2) combined murine antigen-binding loops and human variable region framework residues and IgG1 constant domains. The product trastuzumab (Herceptin), a humanized monoclonal antibody specifically targeting the extracellular domain of the HER2 receptor, was launched in 1998 after approval by the US Food and Drug Administration (FDA). There are several proposed mechanisms of trastuzumab action including inhibition of HER2 shedding, inhibition of PI3K-AKT pathway, inhibition of cyclin E/cdk2 complex activity, attenuation of cell signaling, antibody-dependent cellular cytotoxicity, and inhibition of tumor angiogenesis [95, 96].

4.2. Efficacy and Safety of Trastuzumab. Following preclinical testing, the first clinical evidence of anti-HER2 targeted therapy was provided by phase II trials reported by Baselga et al. [97]. The study was performed in 46 patients with metastatic breast cancer with HER2 protein overexpression with at least 25% of tumor cells exhibiting membrane staining as measured by IHC. All patients were given single-agent therapy with trastuzumab. The overall response rate (complete and partial remission) in assessable patients (n = 43) was 11.6%. Additionally, 37% of the patients achieved minimal responses or stable disease. These results were confirmed by larger multinational clinical trial involving 222 women with HER2-positive metastatic breast carcinoma that had progressed after chemotherapy. After treatment with trastuzumab monotherapy, the overall response rate was 15% (8 complete and 26 partial responses) with a median duration of response of 9.1 months [98]. In another study by Vogel et al., trastuzumab was given as first-line treatment in 114 randomized HER2-positive breast cancer patients with metastatic disease [99]. The overall response rate in this group was 26%. More significantly, the response rate in tumors with 3+ staining by IHC (strong complete membrane staining in >10% tumor cells) was 35% compared to absence of response in tumors with 2+ staining (weak to moderate complete membrane staining in >10% tumor cells). The response rate in tumors with HER2 gene amplification by FISH was 34% compared to 7% in tumors that were negative by FISH [99].

Phase III trials were reported by Slamon et al., where 469 women with progressive metastatic HER2 positive breast cancers were randomly assigned into two groups [100]. The first group (n = 234) received standard chemotherapy alone, and the second group (n = 235) received standard chemotherapy plus trastuzumab. Patients who received chemotherapy with trastuzumab showed longer time to disease progression (median, 7.4 versus 4.6 months), higher rate of response (50% versus 32%), longer duration of response (median, 9.1 versus 6.1 months), lower rate of death at 1 year (22% versus 33%), longer survival (median, 25.1 versus 20.3 months), and a 20 percent decrease in risk of death [100]. Favorable clinical outcome was also noted when trastuzumab combined with Paclitaxel was administered after doxorubicin and cyclophosphamide to patients enrolled in National Surgical Adjuvant Breast and Bowel Project (NSABP) B-31 and the North Central Cancer Treatment Group (NCCTG) N9831 trials with surgically removed HER2-positive breast cancers [101]. Similar results have been reported by other phase III trials evaluating response of HER2-positive breast cancers treated with neoadjuvant chemotherapy and trastuzumab [102–104].

During the clinical trials of trastuzumab, it was observed that a small proportion of patients developed cardiotoxicity manifested as congestive heart failure, cardiomyopathy, and/or decrease in ejection fraction [98]. Occurrence of these unexpected adverse events prompted a retrospective review of all patients enrolled in seven phase II and III trials. The analysis revealed increased risk of developing cardiac dysfunction in patients receiving trastuzumab [105]. The severity was observed to be greatest in patients receiving trastuzumab with anthracycline and cyclophosphamide (27%), compared to those receiving trastuzumab and paclitaxel (13%) or trastuzumab alone (3%–7%). It was also noted that though the cardiac dysfunction was symptomatic in most patients (75%), standard treatment for congestive heart failure led to improvement in most patients (79%). Overall, it was concluded that in spite of these adverse effects treatment was justified in patients with metastatic breast cancer due to the improved overall survival following therapy [105].

4.3. Newer HER2-Targeting Drugs. Lapatinib (Tykerb) is a novel reversible dual inhibitor of HER2 and EGFR tyrosine kinases [106, 107]. The antitumorogenic properties of this drug was examined in human normal and tumor-derived cell lines by in vivo and in vitro studies [108] and in patients with advanced malignancies [109]. Lapatinib was approved by the FDA in 2007 for use in previously treated advanced metastatic breast cancers which overexpressed HER2 in combination with Capecitabine [110]. In a randomized phase III clinical trial, 324 women with previously treated locally advanced or metastatic HER2-positive breast cancer were assigned to receive Lapatinib with Capecitabine or Capecitabine alone [111]. The patients with combination therapy had 49 (30%) disease progression events compared to 72 (45%) events with monotherapy. Additionally, the median time to progression was 8.4 months for patients receiving combination therapy compared to 4.4 months in patients receiving monotherapy [111]. In a similar phase III trial of 399 women, addition of Lapatinib showed prolongation of time to progression and a trend towards improved overall survival [112].

Other HER2-targeting agents which are still being developed and are in preclinical testing stages include Pertuzumab (Omnitarg), which binds HER2 and sterically hinders the recruitment of HER2 into heterodimers [113], and Ertumaxomab, a bispecific antibody targeting HER2 and CD3 [114].

Targeted therapy with MDX-H210 [115] and 2B1 [116] have shown limited response in initial clinical trials.

5. 2007 ASCO Update of HER2 as Marker for Breast Cancer

The American Society of Clinical Oncology (ASCO) published an update of recommendations for use of HER2 as a marker for breast cancer [117]. According to these updated guidelines, HER2 should be evaluated in every primary invasive breast cancer either at the time of diagnosis or at recurrence in order to guide selection of trastuzumab for treatment. Recommendations were also made regarding utility of HER2 to predict response to specific chemotherapeutic agents. It was suggested that if chemotherapy was considered in a patient with HER2-positive breast cancer, an anthracycline should be considered. For trastuzumab-based therapy, it was suggested that a nonanthracycline regimen may produce similar outcome. The benefit of taxane-based chemotherapy was considered controversial, and use of HER2 to guide its use was not recommended.

6. HER2 Testing

The importance of HER2 as a prognostic, predictive, and therapeutic marker in invasive breast cancer is well recognized, and therefore, it is critical to validate and standardize testing techniques in order to make an accurate assessment of HER2 status. The significant contradictions in various studies can at least in part be attributed to differences in HER2 testing and interpretation [118–121]. Techniques which have been used to assess HER2 protein overexpression are immunohistochemistry, ELISA analysis of tumor cytosols or serum, and Western blot, and methods used to evaluate HER2 gene amplification include Southern blot, slot blot, CISH, FISH, and PCR [22].

Use of solid matrix blotting techniques like Southern blot, slot blot, and especially Western blot are significantly limited due to the dilutional artifacts in the tumor sample. In breast cancer specimens, these artifacts may be composed of benign breast ductal cells, acini, stromal cells, inflammatory cells, and vascular structures resulting in false negative cases [120–122]. Additionally, false positive results may be obtained due to inclusion of in situ carcinoma which can express high levels of HER2 [123–126]. In addition, these techniques need a large amount of tissues which would not be available in biopsy specimens. PCR is a sensitive technique; however, it is also affected by dilutional artifacts, and the analysis is time consuming and labor intensive [120]. The absence of simultaneous morphological assessment in the above studies is also a significant disadvantage.

Contrary to the above, analysis by IHC and FISH can be automated and allow the simultaneous assessment of tumor morphology while eliminating difficulties with dilution artifacts.

6.1. HER2 Immunohistochemistry. IHC analysis of HER2 is a simple-to-perform, widely available and inexpensive test. It is nevertheless affected by several variables including tissue-fixation methods, reagents, assay protocols, antibody sensitivities and specificities, and scoring systems [118, 127–129]. In general, testing of freshly frozen tissues is more reliable than paraffin-embedded tissues as formaldehyde causes cross linking of proteins hindering the access of antibody to the epitope [118, 122, 130]. However, practically, it is not possible to have fresh tissues available in all cases especially when testing at reference laboratories and analyzing archival tissues.

The reagents and antibodies used in an assay are other critical factors. Antibodies differ in their sensitivity to detect HER2 epitopes. The important considerations in an assay are the type of antibody used, clonality of the antibody (monoclonal versus polyclonal), and the dilution factor used. Studies comparing different antibodies have shown marked variation in HER2 detection [118, 131, 132]. Press et al. conducted a study analyzing sensitivity and specificity of 7 polyclonal and 21 monoclonal anti-HER2 antibodies on paraffin-embedded tissues of 187 breast cancers with known HER2 protein overexpression and gene amplification analyzed by Northern blot, Western blot, IHC, and Southern blot performed on frozen tumor specimens [118]. The sensitivity of the antibodies ranged from 6% to 80% and none of the antibodies were able to detect all the cases of breast cancer with HER2 overexpression. In a recent study with the help of College of American Pathologists (CAP), HER2 proficiency was evaluated with use of HER2 peptide analyte controls. Of the 109 participants, who returned evaluable stained slides, suboptimal staining was identified in 20 (18.3%) cases. The causes of failure in these cases were antigen retrieval errors (35%), antibody or staining protocol problems (20%), or a combination of both (45%) [133].

Several studies have shown correlation between membrane-staining pattern of HER2 and protein overexpression [118]. Though cytoplasmic staining can be recognized in cases of breast cancer, it has not been shown to correlate with gene amplification [118], HER2 mRNA levels [134, 135], or have an association with poor prognosis in a subset of node-positive women [34]. One study, however, has shown an association between moderate to strong cytoplasmic staining of HER2 with poor prognosis [136]. Another limitation of IHC scoring system is interobserver variability, particularly in cases with moderate (2+) membrane staining [137, 138].

The two FDA-approved IHC-based tests for testing HER2 overexpression are HercepTest (Dako, Carpinteria, CA) which uses A085 polyclonal antibody and Pathway (Ventana, Tucson, AZ) which uses 4B5 monoclonal antibody. The overall concordance between DAKO HercepTest and clinical trial assays (CTA) in 548 breast tumor specimens was 79% [139]. However, a 2+ score by HercepTest did not correlate well with the CTA, where approximately 42% of cases with HercepTest 2+ score were negative by CTA (0–1+) [139]. The low specificity of HercepTest was also highlighted by other studies [132, 140]. The Pathway kit was first introduced in 2002 when it used a monoclonal antibody CB11. This antibody was replaced by a new monoclonal antibody 4B5 in 2008, which showed sharper membrane

staining and less background staining when compared to CB11 and a higher correlation with FISH with an excellent interlaboratory reproducibility when evaluated in a total of 322 breast cancer patients [141].

6.2. HER2 Fluorescent In Situ Hybridization. FISH is a more reliable, reproducible, sensitive, and accurate procedure which is less affected by tissue fixation and analytical variables compared to IHC. It also offers the benefit of simultaneous evaluation of morphology and gene amplification. Relative to solid matrix blotting procedures, analysis of HER2 gene amplification by FISH showed a sensitivity of 98% and specificity of 100% [142]. The technique, however, is more complex and labor intensive than IHC.

The FDA-approved FISH-based tests for HER2 amplification are PathVysion (Abbott Molecular, Des Plaines, IL), INFORM (Ventana, Tucson, AZ) and HER2 FISH pharmDx (Dako, Carpinteria, CA). The PathVysion HER2 probe kit is a dual color FISH (D-FISH) assay which uses probes targeting HER2 gene and chromosome 17 centromere. The HER2 gene amplification is calculated based on the ratio of HER2 gene copies per chromosome 17 copy number. On the other hand, the INFORM assay is a single-color FISH (S-FISH) assay with a HER2 probe alone. In this assay, the HER2 gene amplification is calculated as an absolute value of HER2 gene copy number per tumor nucleus.

Several studies have assessed the use of tissue microarrays as an efficient method to analyze HER2 gene amplification by FISH in a high-throughput manner [143–145].

6.3. Concordance between FISH and IHC. In general, there is concordance between tumors scored as 3+ by IHC and FISH, while cases scored 2+ by IHC showed the most discrepancy [146–154]. Correlation studies in 2279 cases with invasive breast carcinoma showed a concordance of HER2 status between IHC and both D-FISH (87%) and S-FISH (86%) [155]. Specifically, excellent concordance was seen in groups scored 0, 1+, and 3+ by IHC for both D-FISH (97%) and S-FISH (96%), while the most discordant category was the group scored 2+ [155].

In a multicenter study involving 426 women with breast carcinoma being considered for trastuzumab study, the correlation of IHC by HercepTest and FISH by PathVysion was analyzed [156]. It was found that only 2/270 (0.7%) of IHC 0 or 1+ cases were FISH positive and 6/102 (5.9%) IHC 3+ cases were FISH negative. Of the 54 cases with 2+ staining, only 26 (48%) showed HER2 gene amplification by FISH [156]. Several other studies have also shown absence of gene amplification in subset of cases which were scored 2+ by IHC [147, 149, 153, 154, 157, 158]. Hence, a combined approach with IHC and FISH analysis was recommended for accurate HER2 testing particularly for cases with moderate staining with IHC [137, 148, 149, 152, 157].

In a study evaluating clinical outcomes of 799 patients enrolled in 3 clinical trials with 2+ and 3+ scoring on IHC, it appeared that clinical benefit from trastuzumab therapy was restricted to patients with FISH positive (78%) metastatic breast cancers with higher overall response rate and longer duration of survival when compared to FISH negative (22%) patients [159]. Hence, they concluded that analysis by FISH is a preferred method to select patients for trastuzumab therapy [159]. Other studies have also suggested the use of FISH as a superior method which should be done as the first line of HER2 status assessment [160–162] or at least in all cases scored 2+ or 3+ by IHC [163, 164]. In contrast to the above, an analysis of 2963 breast cancer specimens obtained from 135 hospitals and cancer centers showed that the FISH test had a significantly higher failure rate (5% versus 0.08%), reagent cost ($140 versus $10), longer testing time (36 hours versus 4 hours), and interpretation time (7 minutes versus 45 seconds) in comparison to IHC testing [165]. It was concluded that HER2 status determination is most effective by using IHC as the methods of choice and performing FISH in cases with moderate (2+) staining [165].

7. Current Issues with HER2 Testing

Several studies have identified a subset of false positive breast cancers that are IHC 3+ and negative by FISH ranging from 3% to 22% of all positive cases [146, 148, 153, 156–158, 160]. These inconsistencies may be due to several causes including variability in tissue fixation and processing, intratumoral heterogeneity, and polysomy of chromosome 17 [166, 167].

7.1. Effect of Polysomy 17 on HER2 Testing. Polysomy of chromosome 17 is frequent, and depending on the definition of polysomy, it may be seen in 20%–30% of invasive breast carcinomas [168–171]. Analysis of polysomy 17 requires the use of dual color FISH, and its presence can complicate accurate assessment of HER2 status [172]. Studies have shown polysomy 17 as a contributing factor in a small subset of tumors, which were IHC3+ but lacked HER2 gene amplification [166, 169, 171].

While some studies have shown an association between unamplified polysomy 17 tumors with IHC 3+ protein expression and adverse prognostic features [173], these observations have not been validated by others [170, 174]. A study by Hofman et al. reported a response to trastuzumab monotherapy in FISH-negative tumors with polysomy 17 [175]. However, in a recent study involving 405 patients with metastatic breast cancer, it was observed that polysomy 17 in absence of HER2 amplification did not predict the response to Lapatinib with Paclitaxol compared to paclitaxel alone [176].

A recent analysis of HER2 status by array comparative genomic hybridization in breast carcinoma samples ($n = 97$) has shown that polysomy 17 is a rare event and suggest that the cases detected by FISH represent amplification of chromosome 17 centromere rather than true polysomy [177].

7.2. Intratumoral Heterogeneity. Another pitfall in accurate HER2 status determination and discordance between FISH and IHC is the presence of intratumoral heterogeneity. Several studies have reported the presence of intratumoral heterogeneity of HER2 in breast cancers [178–181], which

may reflect genetic divergence in the tumor cells during clonal evolution [182]. Intratumoral heterogeneity can also contribute to discordance in results between primary and asynchronous metastatic and recurrent tumors [180, 183], synchronous metastatic tumors [184] and small biopsy specimens [180, 181].

A study analyzing HER2 protein expression in patients with locally advanced breast cancers who received neoadjuvant chemotherapy ($n = 39$) and patients who did not receive chemotherapy ($n = 60$) reported that the HER2 IHC scores significantly reduced in patients who received therapy (28.5%) compared to those who did not (11.7%) [185]. In contrast, examination of HER2 amplification in needle core biopsies and subsequent excisions of 100 patients showed excellent concordance, even in a subset of patients who received neoadjuvant therapy, suggesting that heterogeneity is not a significant confounding factor when analyzing HER2 by FISH [186].

In 2008, the CAP/American College of Medical Genetics Cytogenetics resource committee panel defined and provided practice guidelines for breast tumors with genetic heterogeneity [187]. Genetic heterogeneity of HER2 is defined as presence of greater than 5% but less than 50% of infiltrating tumor cells with a HER2/CEP17 ratio of greater than 2.2 [187]. Currently, the clinical significance of genetic heterogeneity and possible benefit from anti-HER2 therapy is not known and additional clinical trials are required.

8. Newer Modalities of HER2 Testing

8.1. Chromogenic In Situ Hybridization. In 2008, FDA-approved SPOT-Light HER2 CISH assay (Invitrogen, Carlsbad, CA) which uses formalin-fixed paraffin-embedded sections and can be used to detect HER2 as a primary test or as a reflex test in IHC equivocal (2+) cases. Amplification by this method is defined as HER2 gene enumerated as greater than 5 dots, clusters (small or large), or a combination per nucleus in a majority (>50%) of carcinoma cells [188]. This is further categorized into low and high amplification. Nonamplification is defined as 1–5 dots of HER2 gene per nucleus present in a majority (>50%) of carcinoma cells [188].

Tanner et al. first described the utility of CISH as an alternative to FISH [189]. A high concordance between FISH and CISH has been established by several other studies [190–194]. In a recent study involving 226 consecutive cases of invasive breast carcinomas obtained from two institutions, tissues were evaluated for HER2 protein expression and amplification by IHC (HercepTest), FISH (PathVysion), and CISH (SPOT-Light) [195]. They compared the results between FISH and CISH using the manufacturer's criteria (nonamplified and amplified) and the ASCO/CAP criteria (nonamplified, equivocal, and amplified). The concordance between CISH and FISH for positive and negative results was 98.5% and 98.6% at the two institutions using the manufacturers' criteria and 99% and 99.1% using the ASCO/CAP criteria [195]. The advantages of CISH include ability to analyze the test by light microscopy, preservation of morphologic features, permanent signals which will not fade with slide storage, lower reagent costs, and need for less expertise than FISH [193, 196].

8.2. Metallographic In Situ Hybridization. Silver In Situ Hybridization (SISH) is an automated enzymatic metallographic ISH technique that is based upon deposition of silver at the target site following an enzymatic reaction. The signals are permanent and can be assessed by bright field microscopes. In a multi-institution study of 298 invasive breast carcinomas, concordance between HER2 gene amplification by SISH and FISH was 96.6% when analyzed by FDA approved criteria and 98.9% when analyzed by ASCO/CAP guidelines after excluding equivocal cases [197]. In addition, the study showed high interobserver reproducibility. Other studies have also shown SISH to be an accurate method to detect gene amplification in paraffin-embedded formalin-fixed tissue [198, 199] and cytology preparations [200].

Other bright field metallographic techniques which have been studied for analyses of HER2 status include gold-facilitated in situ hybridization [201] and EnzMet GenePro which allows simultaneous detection of HER2 gene status by deposition of silver and protein expression [202].

8.3. Brightfield Double In Situ Hybridization. Brightfield Double In Situ Hybridization (BDISH) is a recently described automated technique which utilizes two probes targeting HER2 gene and chromosome 17 centromere (CEN 17) and allows simultaneous analysis of morphological features by a brightfield microscope [203]. Their analysis of 94 breast cancer cases demonstrated a high concordance between HER2 FISH and BDISH using the historical scoring method (98.9%) and the ASCO/CAP criteria including the FISH equivocal cases (95.7%) and after excluding the FISH equivocal cases (100%) [203].

9. Current ASCO/CAP Guidelines for HER2 Testing and Interpretation

Accurate assessment of HER2 status is critical in management of patients with invasive breast cancer. In an attempt to standardize HER2 testing and to improve the accuracy and reproducibility of the test results, the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) panel has made recommendations for HER2 interpretation and testing [204]. The panel recommended determination of HER2 status in all cases of invasive breast carcinoma. Algorithms for interpreting HER2 gene amplification by FISH and protein expression by IHC are provided. The guidelines by ASCO/CAP define an HER2 IHC staining of 3+ as uniform intense membrane staining in >30% of invasive tumor cells as compared to previously defined >10% strong staining. Cases with weak to moderate complete membrane staining in at least 10% of cells are considered equivocal (2+), and in these cases, HER2 gene amplification with fluorescent in situ hybridization (FISH) should be tested. For FISH, the tumor is negative for HER2 gene amplification if the ratio of

HER2 gene signals to chromosome17 signals is <1.8 or HER2 gene copy number is <4.0, equivocal when the ratio is 1.8–2.2 or HER2 gene copy number is 4.0–6.0 and positive if the ratio is >2.2 or HER2 gene copy number is >6.0. Guidelines for tissue processing include keeping the time from tissue acquisition to fixation as short as possible and fixation in 10% neutral buffered formalin for 6–48 hours. Additional guidelines for optimal test validation, internal quality assurance procedures, external proficiency assessment, and laboratory accreditation are also provided.

9.1. Impact of New ASCO/CAP Guidelines. Studies analyzing the impact of the new ASCO/CAP guidelines have shown an improved concordance between IHC and FISH results, improved accuracy, and decrease in number of inconclusive FISH tests after raising the cutoff level to greater than 30% invasive tumor cells for HER2 3+ tumors [205–207]. Other studies have additionally shown decrease in interobserver variability by application of the new criteria [208]. In another study, however, there was no change in concordance between FISH results and IHC3+ cases and all the 27 cases scored as 3+ by IHC remained 3+ after using the new threshold [209]. In our retrospective study, 12 (8.5%) of 141 cases had 11%–30% of invasive tumor cells with intense membrane staining which would have their status changed from 3+ to 2+ (equivocal) based on the new guidelines [210]. The overall concordance between FISH and IHC was improved; however, up to 3% of patients would be disallowed from receiving anti-HER2 therapy based on the new guidelines. Thus, the important question remains whether improved concordance translates into better prediction of response to anti-HER2 therapy. This is also critical in light of recent data, which demonstrated benefit of trastuzumab in patients with HER2 overexpression (IHC 3+) regardless of whether there was evidence of gene amplification [211, 212]. A retrospective analysis of 2268 patients from N9831 adjuvant trastuzumab phase III trial where enrollment was based on previous criteria of HER2 IHC > 10% (3+) or FISH ≥ 2.0 showed that a small percentage (1.5%) of patients eligible for trastuzumab therapy under FDA-approved definitions would not be eligible by the new ASCO/CAP guidelines. Additionally, the trastuzumab effect appeared similar for HER2-positive patients regardless of ASCO/CAP or FDA-approved guidelines [213].

10. Conclusions

In conclusion, the confirmed clinical advantages of HER2-targeted therapy in patients with HER2-positive disease necessitate that all patients continue to be tested for HER2 status on diagnosis [204, 211]. When conducting HER2 testing, we should be aware of various analytical and clinical factors that may affect the testing results and the clinical significance of false positive or negative results.

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Review Article

Breast Tumor Angiogenesis and Tumor-Associated Macrophages: Histopathologist's Perspective

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Much progress has been made since the conceptualization of tumor angiogenesis—the induction of growth of new blood vessels by tumor—as a salient feature of clinically significant primary or metastatic cancers. From a practicing histopathologist's point of view, we appraise the application of this concept in breast cancer with particular reference to the evaluation of proangiogenic factors and the assessment of new microvessels in histopathological examination. Recently, much focus has also been centered on the active roles played by tumor-associated macrophages in relation to tumor angiogenesis. We review the literature; many data supporting this facet of tumor angiogenesis were derived from the breast cancer models. We scrutinize the large body of clinical evidence exploring the link between the tumor-associated macrophages and breast tumor angiogenesis and discuss particularly the methodology and limitations of incorporating such an assessment in histopathological examination.

1. Introduction

Angiogenesis, the growth and remodeling of new blood vessels, is one of the hallmarks of cancer. Acquiring proangiogenic phenotype, tumor cells produce and release proangiogenic factors to initiate angiogenesis whereby the ensuing tumor growth, invasion, and metastasis take place. Subject to this angiogenic switch tenet for its progression, breast cancer has been shown to produce a number of proangiogenic factors. Studies have demonstrated that the evaluation of these proangiogenic factors carries predictive and prognostic values [1, 2]. Prognostic significance of tumor angiogenesis has also been highlighted in clinical studies where higher microvessel densities correlate with poorer survival outcome [3]. Via the control of angiogenesis, another dimension in therapeutic intervention is now unfolded.

In relation to tumor angiogenesis, recent research also focuses on the role of tumor microenvironment. Tumor-associated macrophages, a major component in the leukocytic infiltration in tumor, have aroused much research interest since the propositions of their active involvement in tumor progression [4, 5]. Best summarized as M2 phenotype, tumor-associated macrophages show anti-inflammatory and tumor-promoting characteristics, especially in relation to tumor angiogenesis. Apart from the *in vitro* and *in vivo* animal studies based on the breast cancer models, there is accumulating evidence from the clinical studies that suggests tumor-promoting features of tumor-associated macrophages in breast cancer [6, 7].

In this paper, we outline the conceptual development of breast tumor angiogenesis and evaluate the methodology and limitations of quantifying proangiogenic factors and microvessel density in the assessment of tumor angiogenesis in breast cancer. We summarize the pertinent experimental and clinical data exploring the link between the tumor-associated macrophages and breast tumor angiogenesis, emphasizing the methodology and limitations of histopathological assessment in this regard.

2. Breast Cancer and Tumor Angiogenesis

2.1. Tumor Angiogenesis Is One of the Hallmarks of Cancer. Cancer development and progression is a complex multistep process where novel capabilities, the hallmarks of cancer, are acquired through the accumulation of multiple genetic alternations. These hallmarks of cancer include not only the

tumor's cellular autonomy such as self-sufficiency in growth signals and limitless replicative potential but also the abilities to interact with the surrounding stroma such as development of sustained angiogenesis [8]. In particular, the ability to activate angiogenesis plays a crucial role in controlling tumor progression because tumor growth, invasion, and metastasis are angiogenesis dependent [9].

Folkman first proposed angiogenesis dependency of tumor growth and metastasis in 1971. He hypothesized that tumor would remain in dormancy at a microscopic size (1-2 millimeter) in the absence of angiogenesis [10]. This is comprehensible because a tumor, similar to its non-neoplastic counterpart, requires adequate supply of oxygen and nutrients and an effective means to dispose its waste products for survival and growth; these metabolic needs can be fulfilled through tumor-induced angiogenesis [11]. In fact, all mammalian cells including the tumor cells are restricted to within 100–200 μm of a capillary blood vessel due to oxygen diffusion limit of about 100 μm [12].

In addition, angiogenesis facilitates metastasis. The newly formed tumor blood vessels are structurally abnormal. For instance, increased numbers of fenestrations, vesicles, and vesicovacuolar channels and a lack of normal basement membrane are common in tumor vessels [13]. These abnormal blood vessels are consequently more permeative and would constitute the easier entry point for tumor cells to enter into the circulation and hence distant micrometastases [14]. The ensuing micrometastases at ectopic places would remain dormant unless secondary angiogenesis occurs and paves the way for the establishment of a clinically evident disease [15].

Angiogenic switch concept has been postulated to explain the mechanism underlying tumor dependence on angiogenesis to escape from dormancy. Under this concept, the balance of proangiogenic and antiangiogenic factors would ultimately determine the activation status of the switch. When the balance is tilted towards the angiogenic end, the angiogenic switch is turned on; transition from the avascular phase into the vascular phase will be triggered, permitting exponential tumor growth and subsequent transformation into an aggressive phenotype [16].

Factors regulating this angiogenic switch have been extensively explored across various tumors. More than 40 endogenous proangiogenic and antiangiogenic factors are now known [17].

2.2. Immunohistochemical Evaluation of Proangiogenic Factors Produced by Breast Cancer Needs Validation. Subject to the angiogenic switch tenet for its progression, angiogenesis has been shown by studies to be initiated in the hyperplastic state and to intensify towards the invasive carcinoma end of spectrum in breast cancer [18–20]. Breast cancer has been shown to express at least six different proangiogenic factors. These include vascular endothelial growth factor (VEGF) and its four isoforms (121, 165, 189, and 206 amino acids), transforming growth factor (TGF)-β1, pleiotrophin, acidic and basic fibroblast growth factor (FGF), placental growth factor, and thymidine phosphorylase (platelet-derived endothelial cell growth factor) [21].

Among these factors, VEGF and associated factors have been the centre of many studies addressing the clinical significance of proangiogenic factors in terms of predictive and prognostic values. In predicting the response to chemotherapy or tamoxifen, higher level of VEGF in tumor by immunohistochemistry or in tumor cytosol by EIA/ELISA method forecasted poorer response in a number of studies [1]. In a review of breast cancer patients, an overwhelming 81% of 37 clinical studies demonstrated that the VEGF level in tumor or serum, as determined by either immunohistochemistry or ELISA method, serves as an adverse prognostic marker for disease-free or overall survival [2].

Selected studies within the last 10 years exploring the prognostic significance of VEGF and associated factors in breast cancers using immunohistochemistry methodology are highlighted in Table 1, considering immunohistochemistry as a part of routine histopathological examination. As shown in Table 1, many recent studies performing immunohistochemistry in evaluating the expression of VEGF and associated factors failed to demonstrate the prognostic values of these factors in terms of disease-free survival or overall survival [22–37]. Some studies showed that these proangiogenic factors act as a poor prognostic marker but lose their significance in multivariate analysis [38–48]. A number of limitations in the immunohistochemistry methodology could account for these observations. Morphometric assessment is inevitably subject to the individual evaluator's subjectivity. In addition, there is no validated uniform scoring system employed in the reported studies. Primary detecting antibodies from various sources in these studies would give rise to variable detection sensitivity and specificity of the targeted proangiogenic factors. The establishment of a validated immunohistochemistry evaluation is therefore essential to gain comparable data across clinical studies. In addition, this is particularly relevant if the pathology reporting of breast cancer is to incorporate information regarding proangiogenic factors for therapeutic consideration in view of availability of antiangiogenic therapy in on-going clinical trials.

2.3. Methodological Inconsistency in the Assessment of Tumor Vascularity in Breast Cancer Limits Its Clinical Prognostic Value. Apart from the evaluation of the regulating factors in the angiogenic switch, the quantification of angiogenesis in breast cancer per se has its own clinical prognostic values. In a landmark paper, Weidner et al. demonstrated that by immunostaining the blood vessels, the number of microvessels per 200x field in the highest neovascularization areas (hot spots) correlated with distant metastasis in breast cancer patients, corresponding to a 1.17-fold (95% CI = 1.02, 1.34) increase in the distant metastasis risk for every increase in 10 microvessels [49]. Since then, microvessel density determined by this method and its variants has become the most popular surrogate marker in assessing angiogenesis across various cancers [50]. In breast cancer, higher microvessel densities predict higher risk of subsequent *in situ* cancers and invasive recurrence of previous *in situ* cancers [51], poorer response to treatment [52], and higher occurrence of micrometastases [53–55]. In a meta-analysis of 25 independent studies, high microvessel density

Table 1: Summary of the selected studies in the last 10 years exploring the prognostic significance of VEGF and associated factors using immunohistochemistry in breast cancers.

Patients	Assessment of VEGF expression	Prognostic value of VEGF expression
98 stage II ductal breast cancers [26]	Antibody: monoclonal anti-VEGF165 Scoring system: 0 = none, 1 = <33%, 2 = 33–66%, 3 = >66% positive tumor cells	VEGF had no prognostic significance for overall survival or disease-free survival
48 triple negative breast cancers not receiving systemic adjuvant treatment from 500 primary breast cancers using tissue microarrays [24]	Antibody: polyclonal anti-VEGF Scoring system: cytoplasmic staining intensity was scored from 0 to 3 High expression had score 3	VEGF had no prognostic significance for 5-year breast-cancer-specific survival
125 stage II node-positive invasive ductal carcinomas, NOS 25 stage II node-positive invasive lobular carcinomas [23]	Antibody: polyclonal anti-VEGF-C Staining was graded as strong, medium, or weak-to-absent expression	VEGF-C had no prognostic significance for overall survival or disease-free survival
172 primary breast cancer [25]	Antibody: anti-VEGF-A Scoring system: staining intensity was graded from 0 (negative) to 3 (strong intensity) Positive cases had score 1–3	VEGF-A had no prognostic significance for recurrence-free survival
116 invasive ductal breast cancers [27]	Antibody: anti-VEGF Scoring system: positive cases had >10% positive tumor cell staining	VEGF-A had no prognostic significance for overall survival in multivariate analysis
52 infiltrating ductal carcinomas, 4 intraductal carcinomas, 3 mucinous adenocarcinomas,1 medullary carcinoma, 1 inflammatory breast carcinoma [38]	Antibody: anti-VEGF-C, anti-VEGF-D Scoring system: sum of staining intensity (0 = negative to 3 = strong) and percentage of positive cells (0 = 0%, 1 = 1–10%, 2 = 11–30%, 3 = 31–50%, 4 = 51–100%) High-expression group had score 4–7	High expression of VEGF-C/D had poorer disease-free survival and overall survival
59 invasive ductal carcinomas, NOS 11 other types of invasive breast cancer [39]	Antibody: polyclonal anti-VEGF-C Scoring system: negative, 1+ (focal expression in <5%), 2+ (focal expression in 5–20%), 3+ (diffuse expression in >20%) High-expression group had score above 2+	Shorter disease-free survival and overall survival for high expression of VEGF-C in univariate analysis
215 high-risk primary breast cancers with extensive axillary involvement [28]	Antibody: monoclonal anti-VEGF Staining intensity was graded from 0 to 3+ Positive cases are those having any tumor areas with positive staining	VEGF had no prognostic significance for overall survival or relapse-free survival
177 invasive breast cancers [40]	Antibody: monoclonal anti-VEGF-A, anti-VEGF-D, polyclonal anti-VEGF-C Scoring system: H score (multiplying percentage of positive carcinoma cells by the staining intensity graded 0 to 3) High-expressing tumors had score above the median score	(1) Shorter overall survival for high expression of VEGF-A in univariate analysis (2) Shorter overall survival and disease-free interval for high expression of VEGF-C in univariate and multivariate analyses (3) No prognostic significance for VEGF-D (4) Tumours with high expression of both VEGF-A and -C had significantly shorter overall survival
130 invasive ductal carcinomas, 30 invasive lobular carcinomas [41]	Antibody: polyclonal anti-VEGF-B, monoclonal anti-VEGF-A (165, 189, 206 a.a.) Scoring system: 0 (no or weak staining in <10%), 1 (weak-to-moderate staining in 11–20%), 2 (moderate-to-strong staining 21–50%), 3 (strong staining in >50%) Positive cases had score above 2	(1) VEGF-A had no prognostic significance (2) Unfavorable disease-free and overall survival for VEGF-B-positive cases in lymph node metastases cases
136 invasive ductal carcinomas, 31 invasive lobular carcinomas [42]	Antibody: polyclonal anti-VEGF-C, polyclonal anti-VEGF-D Scoring system: positive cases had at least 10% immunoreactive tumor cells	Poorer overall survival for VEGF-C-positive cases VEGF-D had no prognostic significance

Table 1: Continued.

Patients	Assessment of VEGF expression	Prognostic value of VEGF expression
80 invasive ductal carcinomas, 15 ductal carcinomas *in situ*, 5 lobular carcinomas *in situ*, 14 invasive lobular carcinomas, 6 medullary carcinomas, 2 tubular carcinomas [43]	Antibody: monoclonal anti-VEGF Scoring system: 0 = none, 1+ = < 5%, 2+ = 5–50%, 3+ = >50% positive tumor cells High reactivity cases had score above median value	Overexpression of VEGF had both unfavorable overall survival and disease-free survival
114 breast cancers [29]	Antibody: monoclonal anti-VEGF165 Scoring system: staining intensity was graded from 0 (no staining) to III (most intense staining)	VEGF had no prognostic significance for disease-free survival or cancer survival
100 invasive ductal carcinomas, NOS, 19 invasive lobular carcinomas [30]	Antibody: polyclonal anti-VEGF-C Staining was graded as strong, medium, or weak expression	VEGF-C had no prognostic significance for overall survival or disease-free survival
323 invasive breast carcinomas [31]	Antibody: monoclonal anti-VEGF Scoring system: sum of staining intensity (0 = negative to 3 = strong) and percentage of positive cells (0 = 0%, 1 = 1–25%, 2 = 26–50%, 3 = >50%) Positive cases had score 4–6	VEGF was not associated with incidence of relapse or death
181 invasive ductal carcinomas, 22 invasive lobular carcinoma, 8 invasive ductal and lobular (mixed) carcinomas, 5 ductal *in situ* carcinomas, 1 medullary carcinoma [32]	Antibody: anti-VEGF-C Scoring system: cytoplasmic staining was graded negative (negative), 1+ (10–39%), 2+ (40–69%), 3+ (>70%)	VEGF-C had no prognostic significance for disease-free survival
238 invasive breast cancers not receiving tamoxifen from 500 primary breast cancers using tissue microarrays [22]	Antibody: polyclonal anti-VEGF Scoring system: cytoplasmic staining intensity was scored from 0 to 3 High staining intensity group had score 3	VEGF had no prognostic significance for relapse-free survival
87 primary breast cancers [33]	Antibody: polyclonal anti-VEGF-C Scoring system: 0 (no staining or cytoplasmic staining in <10%), 1+ (faint cytoplasmic staining in >10%), 2+ (weak-to-moderate complete cytoplasmic staining in >10%), 3+ (strong complete cytoplasmic staining in >10%) Positive cases had score 2+ or 3+	VEGF-C had no prognostic significance for disease-free survival or overall survival
224 invasive breast cancers using tissue microarrays [44]	Antibody: polyclonal anti-VEGF Scoring system: staining intensity was graded from 0 (negative) to 3 (intense intensity), and the percentage of positive cells was recorded (0 = 0%, 1 = <1%, 2 = 1–10%, 3 = 10–50%, 4 = 50–90%, 5 = >90%) Positive cases are those having any positive staining	VEGF-A-positive cases had favorable disease-free survival at 10-year followup in multivariate analysis
207 invasive breast carcinomas [34]	Antibody: polyclonal anti-VEGF-D Scoring system: 0 = negative, 1 = weak focal staining, 2 strong focal/widespread moderate staining, 3 = strong widespread staining Positive cases had score 2 or 3	VEGF-D had no prognostic significance for overall survival or relapse-free survival
96 invasive ductal carcinomas, 9 other invasive carcinomas [45]	Antibody: monoclonal anti-VEGF-D Scoring system: positive cases had more than 10% tumor cells with cytoplasmic staining	(1) Positive VEGF-D cases had poorer disease-free survival in univariate and multivariate analyses (2) Positive VEGF-D cases had poorer overall survival in univariate analysis
228 invasive unilateral breast carcinomas [35]	Antibody: monoclonal anti-VEGF (isoforms 121, 165 and 189) Scoring system: positive cases had more than 1% immunoreactive tumor cells	VEGF had no prognostic significance for overall survival or relapse-free survival
114 invasive ductal carcinomas, 9 other invasive carcinomas [46]	Antibody: polyclonal anti-VEGF-C Scoring system: positive cases had more than 10% immunoreactive tumor cells	Positive VEGF-C cases had poorer disease-free survival and overall survival in univariate analysis

Table 1: Continued.

Patients	Assessment of VEGF expression	Prognostic value of VEGF expression
99 invasive ductal carcinomas, NOS [36]	Antibody: anti-VEGF Scoring system: positive cases had more than 10% tumor cells with membrane or cytoplasmic staining	VEGF had no prognostic significance for overall survival or relapse-free state
107 primary invasive breast carcinomas [47]	Antibody: anti-VEGF-A, anti-VEGF-C, anti-VEGF-D Scoring system: computer-assisted image analysis based on the percentage of immunostained surfaces and mean optical density High-expression group had value equal to or higher than median	(1) High-VEGF-A-expression cases had worser disease-free survival (2) VEGF-C or VEGF-D had no prognostic significance (3) Cases with both low VEGF-A and VEGF-C expression had better disease-free survival
242 node-negative breast cancer [37]	Antibody: polyclonal anti-VEGF isoforms 121, 165, 189, and 206 Scoring system: high-expression cases had >40% immunopositive tumor cells	VEGF had no prognostic significance for disease-free survival or overall survival
94 invasive breast cancer, 4 noninvasive cancer [48]	Antibody: polyclonal anti-VEGF-C Scoring system: positive cases had over 10% tumor cells stained positively	VEGF-C-positive group had poorer disease-free survival

significantly predicted poor relapse-free survival and overall survival (both RR = 1.54, 95% CI = 1.29, 1.84) [3].

However, scrutinizing each of the studies included in the above-mentioned meta-analysis, variations in results regarding prognostic value of microvessel density in breast cancer patients' survival are apparent [3]. The choice of antibodies to highlight the blood vessels in various studies could be a contributing factor because each antibody has its own specificity and sensitivity against the endothelial cells of the blood vessels. Among the commonly used antibodies are antibodies against factor-VIII-related antigen, CD31, and CD34. Anti-CD34 is now considered the optimal marker for its higher sensitivity without high failure rate in antigen retrieval for invasive breast carcinoma studies [3, 56–58].

Another factor to consider in assessing microvessel density is the variations from the original method designed by Weidner et al. These include variables such as the number of hot spots counted, the areas and fields of magnification (magnification of a field area of 200x or 400x), the subjectivity in identification of what constitutes a stained blood vessel, and also the descriptive statistics in reporting the number of microvessel density (the mean or the highest value). To overcome the subjectivity of observers, a 25-point Chalkley microscope eyepiece graticule has been introduced. The graticule is orientated in such a way that it gives the maximum number of graticule points overlapping the highlighted vessels. This method measures relative area and has strong association with vessel area and number [3, 57–59]. Both the conventional optical assessment method and the Chalkley method have been used in studies that demonstrated increased microvessel density as a poor prognostic factor [3, 60, 61]. Controversies over the best methodology remain despite a proposed consensus of using Chalkley method in angiogenesis quantification in solid human tumors [58].

2.4. Antiangiogenic Therapy Gives Promising Results in Preclinical Studies but Not in Clinical Trials of Metastatic Breast Cancer. Given the pivotal roles of proangiogenic factors in tumor angiogenesis, these factors serve as reasonable pharmacological targets for inhibition of tumor angiogenesis. Among these factors, blockage of the VEGF pathways was the focus of many preclinical studies because VEGF is the most potent proangiogenic factor [62].

A number of experimental xenograft models using different tumor cell types including breast carcinomas showed that anti-VEGF therapy resulted in 25% to 95% of tumor growth inhibition in a dose-dependant manner. Functionally, tumor microvascular permeability was also reduced [63]. Upon antiangiogenic drug treatment, tumor vessels remodel and transiently resemble the normal vessels. During this normalization window, the normalized tumor vessels are believed to be more efficient in delivering the nutrients as well as cytotoxic drugs and oxygen, potentiating the effects of the combination of cytotoxic and antiangiogenic therapies targeting the tumor cells and endothelial cells, respectively [64, 65]. This tumor vasculature normalization model provides a rationale for the observed better effects of combined cytotoxic and anti-VEGF therapy as compared to single-agent treatment in preclinical studies [63]. Although this tumor vasculature normalization model is conceptually appealing, histologic examination of vasculature normalization in clinical setting to identify the optimized normalization window is limited in several aspects such as representative multiple small biopsies that would be hardly obtained for the global assessment of the solid tumor [64, 65].

Results from the preclinical studies pave the way for the use of anti-VEGF therapy in clinical trials. However, the results from the recent phase III clinical trials in breast cancer treatment using bevacizumab, a humanized monoclonal

antibody against VEGF, are not as promising as in animal studies. A meta-analysis including five reported clinical trials involving metastatic breast cancer patients showed that the combined bevacizumab and chemotherapy arm had better objective response (RR = 1.26, 95% CI = 1.17, 1.37) and progression-free survival (HR = 0.70, 95% CI = 0.60, 0.82) as compared to the chemotherapy-alone arm. However, no significant advantage was seen with the addition of bevacizumab as compared to the chemotherapy-alone arm for overall survival (HR = 0.90, 95% CI = 0.80, 1.03) [66].

Two trials have published results and one has a report published recently for further inspection of the study design [83–85]. Although bevacizumab specifically blocks the VEGF-mediated pathways, all of these trials used bevacizumab as a general therapy given on a population basis without considering the specific molecular phenotype of the breast cancer. VEGF expression profile of the cancers was not investigated in the enrolled patients and the best methodology of evaluation has not yet been validated. Redundancy of other proangiogenic factors might also play important roles in advanced breast cancer. Consideration in these factors is needed to better stratify the patients who will most likely benefit from the VEGF-targeted therapy.

3. Roles of Tumor-Associated Macrophages in Breast Cancer

3.1. Macrophages Are Recruited into the Tumor. Infiltration of leukocytes in tumors was first recognized by Virchow in 1863 prompting him to postulate the link between the origin of cancer and inflammation [4]. This link, arbitrarily termed the extrinsic pathway, increases the risk of cancer development, exemplified by inflammatory conditions associated with malignancy such as ulcerative colitis linked to the development of colon cancer. In contrast, it is now evident that the intrinsic pathway, genetic alterations causing cancer without casual relationship to inflammatory processes, also leads to a protumor inflammatory microenvironment [86].

Among the heterogeneous populations of the leukocytic infiltrates, it has now been established that macrophages constitute the major proportion; for instance, up to 50% of cell mass in breast carcinoma is composed of macrophages [87]. These macrophages are called tumor-associated macrophages. They are mostly derived from the peripheral blood monocytes and recruited into the tumor by a wide range of chemokines and growth factors released by the tumor cells. Among these, CC chemokines, particularly CCL2 (formally monocyte chemoattractant protein-1 or MCP-1) and CCL5, and growth factors such as colony-stimulating factor-1 (CSF-1) and vascular endothelial growth factor (VEGF) are strongly implicated in macrophage recruitment in various tumors including breast cancer [88].

3.2. Tumor-Associated Macrophages Are Polarized into M2 Phenotype in Tumor Microenvironment. The interaction between the tumor cells and the recruited tumor-associated macrophages has aroused much research study interest. The classical view of tumor-associated macrophages displaying antitumor response to destroy the tumor cells, similar to their pathophysiological response to microbial invasion, has however been confronted by a large number of studies that contradictorily showed their opposite protumor response. This paradoxical function of tumor-associated macrophages in relation to tumor is explained by the macrophage balance hypothesis where the outcomes of the tumor-associated macrophages depend on the polarization between two extremes of a continuum: M1 as proinflammatory and microbicidal/tumoricidal phenotype in contrast to M2 as anti-inflammatory and tumor-promoting phenotype [5].

Clinical studies across various human tumors exploring correlation between tumor-associated macrophage density and prognosis have shown constant strong inverse relationship in carcinomas of breast and cervix but a minority of conflicting results in prostate, stomach, and lung cancers [89]. These results suggest the importance of tumor microenvironment in tilting the macrophage balance and support largely the polarization of macrophage into protumor M2 phenotype by most tumors, including the breast carcinomas.

3.3. Tumor-Associated Macrophages Enhance Tumor Progression in Breast Cancer. In vitro and *in vivo* animal studies, especially the animal model of mammary tumor, have shed much light on the roles of tumor-associated macrophages in tumor progression. For instance, when a null mutation *colony stimulating factor-1* gene was crossed into transgenic mice susceptible to mammary cancer due to the expression of the polyoma middle T antigen oncogene (PyMT mice), depletion of macrophages resulted in delayed tumor progression and tumor metastasis. In contrast, overexpression of *CSF-1* gene resulted in increased macrophage infiltrates and in turn accelerated tumor progression and tumor metastasis [90].

Restricting the data pertinent to human breast cancer, the increased tumor-associated macrophages number correlates with high proliferative activity of the tumor cells as indicated by higher mitotic grade and Ki-67 labelling [6, 69, 76, 77, 81]. This association could be explained by the direct mitogenic stimulation of tumor cells by tumor-associated macrophages or indirect effect via stimulation of tumor angiogenesis by tumor-associated macrophages as discussed below. For the former possibility, tumor-associated macrophages indeed express and release a wide range of growth factors such as epidermal growth factor, basic fibroblast growth factor-2 (FGF-2), transforming growth factor-β, VEGF, and platelet-derived growth factor (PDGF) [91]. In particular, it has been shown that tumor-associated macrophages secrete epidermal growth factor, but the normal or malignant breast cancer cells do not [92]. Many breast cancers express epidermal growth factor receptor [93], which upon activation by this ligand leads to tumor survival and proliferation [94].

Furthermore, a paracrine loop between breast cancer cells and tumor-associated macrophages could promote the

invasion of breast carcinoma via reciprocal stimulation because CSF-1 secreted by breast cancer cells recruits macrophages, and epidermal growth factor derived from the recruited macrophages promotes tumor cell motility [95]. In addition, tumor-associated macrophages produce enzymes and inhibitors, which regulate the digestion of the extracellular matrix such as matrix metalloproteinases (MMPs) [96] and urokinase-type plasminogen activator (uPA) [97]. Accordingly, degradation of the extracellular matrix by these macrophage proteases would facilitate the invasion of tumor cells into the stroma and hence metastasis [89, 98]. This constitutes one of the mechanisms explaining the association of poor prognosis in breast cancer with higher macrophage density in clinical studies [7, 71, 77, 79].

3.4. Tumor-Associated Macrophages Enhance Tumor Angiogenesis in Breast Cancer. As discussed above, tumor angiogenesis is crucial for tumor progression. Tumor angiogenesis was initially thought to be induced only by tumor cells themselves; however, tumor-associated macrophages are indeed a major player in the regulation of tumor angiogenesis [99]. It is now evident that tumor-associated macrophages recruited into the tumor microenvironment are producers of a wide range of proangiogenic factors, including IL-1, VEGF, IL-8, bFGF, and TNF-α [100].

The process of activation and transformation of the tumor-associated macrophages into this proangiogenic phenotype is dependant on several tumor microenvironmental stress factors such as low oxygen, low pH, and high lactate concentration [101]. Tumor hypoxia appears to be the major regulating factor. One study has shown that the median pO2 value in breast cancer was 30 mmHg compared to 65 mmHg in normal tissue and could be as low as between zero and 2.5 mmHg [102]. Macrophages are attracted to these hypoxic areas [70, 73], and via the hypoxia-induced pathway, large numbers of genes encoding the proangiogenic factors are dramatically upregulated in the tumor-associated macrophages [103].

The first clinical study correlating tumor-associated macrophages and angiogenesis also came from a study on breast cancer. Significant correlation between the two was shown in addition to the prognostic value of tumor-associated macrophages, implying the crucial role of angiogenesis driven by tumor-associated macrophages in breast cancer progression [7]. Later clinical studies also produced similar findings [76, 77, 79, 81]. *In vivo* animal study employing PyMT mice showed that the inhibition of macrophage maturation and infiltration into tumors delayed angiogenesis and tumor progression, providing evidence of causal role of tumor-associated macrophages in tumor angiogenesis [104].

3.5. Assessment of Tumor-Associated Macrophages in Breast Cancer by Immunohistochemistry Varies in Clinical Studies. Major findings in recent clinical studies exploring the link between the tumor-associated macrophages and other clinicopathological parameters in invasive breast carcinomas using immunohistochemistry are summarized in Table 2. These studies are generally agreeable in terms of association between the density of macrophages and clinicopathological parameters related to tumor progression. Besides, the significant association between density of macrophages and microvessel density implies the role of tumor-associated macrophages in tumor angiogenesis.

As shown in Table 2, in all but two studies, the antibody against CD68 was used to highlight the presence of macrophages. However, there is variation in the methods used to assess tumor-associated macrophages in these studies. Some studies used semiquantitative methods [68, 76, 78, 79] and others used quantitative methods with variation in selection of fields and count [6, 7, 70–75, 77, 80–82]. These variations in assessment method would give rise to minor discrepancies among the studies. In particular, no much attention was given to the location of tumor-associated macrophages in relation to breast carcinomas. It is known that the tumor-associated macrophages are attracted to hypoxic tumor areas, and angiogenesis is likely to be induced at these hypoxic areas. Most studies used the "hot spot" method to identify the areas of the highest number of tumor-associated macrophages [7, 70–75, 77, 80–82]. These studies most probably have evaluated the tumor-associated macrophages at tumor margin where angiogenesis occurs, as opposed to tumor-associated macrophages within the tumor nest where information regarding their role is still lacking [105]. Evaluation by this "hot spot" methodology could also alleviate the concern about the confounding macrophages induced by biopsies prior to surgical resection of the tumor, as it is unlikely that a biopsy tract would induce accumulation of macrophages only at the tumor margin.

Given the many positive findings regarding the association of macrophages and breast tumor progression, a standardized evaluation method for assessing tumor-associated macrophages is therefore necessary to harmonize future research. A consensus of using "hot spot" method with particular reference to tumor-associated macrophages in tumor stroma would probably constitute such a template for examination.

3.6. Targeting Tumor-Associated Macrophages in Breast Cancer Represents an Attractive Approach. A plethora of growth factors, cytokines, and chemokines are employed in the process of recruitment, survival, activation and polarization, proangiogenic activity, and matrix remodeling of tumor-associated macrophages. These factors represent reasonable therapeutic targets [106]. For instance, in an experimental breast cancer model, antagonizing the chemokine CCL5 receptors expressed on the macrophages reduced the number of tumor-associated macrophages and slowed the tumor growth [107]. Using the anti-VEGF antibody to treat breast cancer xenografts, in addition to the inhibition of angiogenesis, infiltration of tumor-associated macrophages was also reduced. In these studies, tumor growth and distant metastases were inhibited [108, 109]. Although the contribution of reduction of tumor-associated macrophages to the observed results in these experimental studies has yet to be determined, pathological correlation in this aspect in the clinical trials employing anti-VEGF therapy would be of great interest.

Table 2: Summary of clinical studies exploring the link between tumor-associated macrophages and other clinicopathological parameters in invasive breast carcinomas.

Tumor type	Means of tumor-associated macrophages assessment	Findings
101 invasive breast carcinomas [7]	Macrophage marker: CD68 Macrophage index was determined by 25-point Chalkey graticule as the mean of three "hot spot" counts under 250x field	(1) High macrophage index correlated with high vascular grade (2) High macrophage index in poorly vascularized areas (3) High macrophage index predicted reduced relapse-free and overall survival
75 invasive breast carcinomas with lymphoplasmacytic infiltrates [67]	Macrophage marker: CD11c Macrophage was counted as percentage of total leukocyte infiltrate identified by CD45	(1) Macrophage predominance in leukocyte infiltrate correlated with high grade and c-erbB-2 expression
75 invasive breast carcinomas (50 ductal, 9 lobular, 5 mixed, 5 tubular/cribriform, 1 mucinous) [68]	Inflammation was classified as diffuse, perivascular, and perilobular on H&E and also using markers. Intensity was qualitatively graded as from 0 (absent) to 3 (marked) Macrophage marker: CD68	(1) In diffuse inflammation pattern, macrophage intensity predominated other cell types and was associated with high-grade, large tumor size, tumor necrosis, and c-erbB-2 expression (2) Intensity of diffuse inflammation but not macrophage correlated with vascularity
120 invasive breast carcinomas (60% ductal, 20% lobular, 20% others) [6]	Macrophage marker: CD68 (KP-1 antibody) Macrophages were counted in 40 hpf (20 hpf tumor cell zones and 20 hpf stromal zones) and graded from weak (<300) to intense (>500)	(1) Intensity of macrophage was higher in node-negative tumors (2) Intratumoral macrophage infiltration correlated with high tumor grade, absence of ER, and high mitotic grade
57 invasive breast carcinomas NOS (abstract) [69]	Macrophage marker: CD68	(1) Tumor-associated macrophages correlated with mitotic activity index
109 invasive breast carcinomas (ductal 88, lobular 8, others 13) [70]	Macrophage marker: CD68 Macrophage index was determined by 25-point Chalkey graticule as the mean of three "hot spot" counts under 250x field	(1) Higher macrophage index associated with necrosis
26 invasive ductal carcinomas (13 cases <5 years, 13 cases >5 years' survival) [71]	Macrophage marker: CD68 Hot spots were identified under 100x, field and macrophages were counted in 5 hpf under 400x field	(1) Higher macrophage count in poor prognosis group
151 invasive ductal carcinomas [72]	Macrophage marker: CD68 Macrophages were counted in 5 hot spots, and the mean of the highest three was determined (per mm^2)	(1) High macrophage count correlated with high levels of macrophage chemoattractant protein-1 and thymidine phosphorylase in breast cancer by ELISA (2) High level of macrophage chemoattractant protein-1 had worsened relapse-free survival
96 invasive breast carcinomas (78 ductal, 7 lobular, 11 others) [73]	Macrophage marker: CD68 Macrophage index was determined by 25-point Chalkey graticule as the mean of three "hot spot" counts under 250x field	(1) Macrophage index correlated with high VEGF and EGFR expression (2) In EGFR-negative cases, high VEGF correlated with increased macrophage index, high grade, presence of necrosis, and increased tumor p53 expression (3) No significant prognostic value of VEGF
24 invasive breast carcinomas (12 ductal, 12 lobular) [74]	Macrophage marker: CD68 Macrophage index was determined by 25-point Chalkey graticule or by absolute count as the mean of five VEGF positive areas under 200x field. In VEGF-negative areas, 5 most or least vascularized areas were chosen	(1) Macrophage count was higher in less vascularized areas
230 invasive ductal carcinomas [75]	Macrophage marker: CD68 macrophages were counted in 5 hot spots, and the mean of the highest three was determined (per mm^2). Graded from 0 (<50/mm^2) to 2 (>100mm^2)	(1) High macrophage count showed a tendency of correlation with high level of tumoral macrophage chemoattractant protein-1 by immunohistochemistry ($P = .089$). (2) High level of tumoral macrophage chemoattractant protein-1 showed a tendency of correlation with high microvessel density grade ($P = .087$)

Table 2: Continued.

Tumor type	Means of tumor-associated macrophages assessment	Findings
97 invasive ductal carcinomas [76]	Macrophage marker: CD68 Macrophages were semiqualitatively graded as 1 = no macrophages, 2 = small foci of macrophages, 3 = large foci of macrophages 4 = diffuse macrophages infiltration in tumor stroma	(1) Higher macrophage grade associated with higher VEGF expression, higher microvessel density, and higher mitotic activity index
249 invasive ductal carcinomas (abstract) [77]	Macrophage density was assessed as average density of three hot spots at a magnification of 400x	(1) Macrophage density significantly correlated with both the VEGF expression and MVD (2) Macrophage density was associated with the nuclear grade, estrogen receptor status, and MIB-1 count (3) Patients with a high macrophage density had a significantly worse disease-free survival prognosis than those with a low density
97 breast carcinomas [78]	Macrophage marker: CD68 Macrophages were semiqualitatively graded as 1 = no macrophages, 2 = small and large foci of macrophages, 3 = diffuse macrophages infiltration in tumor stroma	(1) Macrophage grade was not correlated with tumor chemoattractant protein-1
78 invasive breast carcinomas (48 ductal, 30 lobular) [79]	Macrophage marker: HAM56 antibody Macrophages were semiqualitatively graded as 1 = no macrophages, 2 = small foci of macrophages, 3 = large foci of macrophages, 4 = diffuse macrophages infiltration in tumor stroma	(1) Higher macrophages in invasive ductal carcinomas compared to invasive lobular carcinomas (2) In invasive ductal carcinomas, macrophage grade correlated with tumor size, lymph node metastasis, stage, microvessel density, VEGF, and tumor grade (3) In invasive ductal carcinomas, macrophage grade and clinical stage were predictive in disease-free survival rate
133 invasive breast carcinomas (94 ductal, 28 lobular, 8 mucinous, 3 papillary) [80]	Macrophage marker: CD68 Macrophages were counted in 5 consecutive 400x fields in areas identified as "hot spots" under 100x	(1) Higher macrophage count associated with high tumor grade, p53 expression, absence of ER, high VEGF expression in macrophage, and postsurgical serum VEGF level
168 invasive primary breast cancer (142 ductal, 20 lobular, 6 others) [81]	Macrophage marker: CD68 Macrophages were counted using point counting method (expressed as percentage of volume occupied by a component out of total volume) using a 100-point ocular grid counting at 400x field over 30 fields and were grouped tertiles	(1) High tertile percentage of macrophage correlated with high tumor grade, high Ki-67 labelling, absence of hormonal receptors, high microvessel density, high CD4 and CD8 count
128 invasive ductal carcinomas [82]	Macrophage marker: CD68 Macrophages were counted as mean of the 3 densest areas at 200x field (per mm^2) following a brief scan at low power and separated into <320 or >320/mm^2 groups	(1) Macrophage count correlated with stromal chemoattractant protein-1 (2) Stromal chemoattractant protein-1 correlated with lymphatic invasion and predicted worsened relapse-free survival

4. Conclusion

In conclusion, the salient points regarding trilateral relationship among breast cancer cells, tumor-associated macrophages, and tumor angiogenesis are

(1) breast cancer progression is dependent on tumor angiogenesis,

(2) breast cancer cells are able to regulate tumor angiogenesis via production of proangiogenic factors,

(3) tumor-associated macrophages have emerged as a major player in regulating breast cancer progression,

(4) as a major regulatory mechanism in tumor progression, tumor-associated macrophages enhance breast tumor angiogenesis,

(5) breast cancer progression involves reciprocal interactions between breast cancer cells and tumor-associated macrophages.

At the tissue level, the assessment of the relationship between these three compartments is feasible by histopathological examination coupled with immunohistochemistry. Despite its limitations, microvessel density has been widely

used as a surrogate marker for tumor angiogenesis. Establishment of a validated immunohistochemical evaluation of proangiogenic factors produced by breast cancer is essential. Information regarding expression profile of proangiogenic factors might help to stratify patients receiving antiangiogenic therapy. Tumor-associated macrophage density can be graded in similar manner as microvessel density evaluation. Assessment in this regard would possibly constitute another important item in histopathological examination for prognostication, considering therapeutic advances targeting tumor-associated macrophages.

Conflict of Interests

All authors have no conflict of interests.

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Review Article

Circulating Tumor Cells in Breast Cancer Patients: An Evolving Role in Patient Prognosis and Disease Progression

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In this paper, we examine the role of circulating tumor cells (CTCs) in breast cancer. CTCs are tumor cells present in the peripheral blood. They are found in many different carcinomas but are not present in patients with benign disease. Recent advances in theories regarding metastasis support the role of early release of tumor cells in the neoplastic process. Furthermore, it has been found that phenotypic variation exists between the primary tumor and CTCs. Of particular interest is the incongruency found between primary tumor and CTC HER2 status in both metastatic and early breast cancer. Overall, CTCs have been shown to be a poor prognostic marker in metastatic breast cancer. CTCs in early breast cancer are not as well studied, however, several studies suggest that the presence of CTCs in early breast cancer may also suggest a poorer prognosis. Studies are currently underway looking at the use of CTC level monitoring in order to guide changes in therapy.

1. Introduction

Breast cancer is one of the most common cancers affecting women. It is estimated that one in eight women will develop an invasive breast cancer at some point during her lifetime. In 2010, according to the American Cancer Society, approximately 207, 090 new cases of invasive breast cancer will be diagnosed and 39, 840 women will die from metastatic disease. In this era of molecular medicine, novel approaches are needed in the management of breast cancer. In the last several decades, circulating tumor cells (CTCs) have emerged as a unique target for understanding disease progression, prognosis, and treatment in breast cancer pathogenesis.

CTCs are tumor cells present in the peripheral blood. They are found in many different carcinomas but are not present in patients with benign disease [1]. Much of the research involving CTCs stems from studies involving disseminated tumor cells (DTCs). DTCs are tumor cells present in the bone marrow. Briefly, several studies have shown that patients with DTCs at the time of diagnosis have larger tumors, higher histologic grade, and a higher incidence of lymph-node metastasis, distance metastasis, and cancer-related death versus those patients without DTCs [2, 3]. Furthermore, detection of DTCs after systemic treatment is associated with increased risk of recurrence and decreased disease-free survival as well as decreased breast cancer-specific survival [4, 5]. Though DTCs have been more thoroughly studied, there are several studies that have documented a correlation between the occurrence of DTCs and CTCs in both primary and metastatic breast cancer [6–10]. Since bone marrow sampling is cumbersome, difficult to reproduce, and morbid for patients, emphasis has been

placed on advancing CTC research. This paper will address the current methodologies of CTC detection, the prognostic role of CTCs in both early and advanced breast cancer, and the implication of CTCs in disease progression, treatment, tumor biology, and further research.

2. Evidence for CTC in Early Metastasis

It was previously thought that metastasis occurred late in disease progression; however, evidence from CTCs/DTCs has shown that metastasis may be an early event. This is supported by the fact that CTCs/DTCs are found in patients with early breast cancer. A recent study by Husemann et al. with transgenic (HER2/PyMT) mice showed that dissemination of tumor cells can occur at a preinvasive stage of the primary tumor. They also found both in mice and early human breast cancer that the presence of CTCs/DTCs was independent of tumor size [11]. However, even though occult tumor dissemination may occur early, not all patients with detectable CTCs/DTCs will develop overt metastases. Meng et al. looked at 36 breast cancer patients 7 to 22 years after mastectomy and found that 36% had evidence of CTCs with no evidence of clinical disease [12]. Similarly, in a large pooled analysis by Braun et al., only half of DTC-positive breast cancer patients relapsed over a ten-year period [3]. These CTCs/DTCs may be in a state of dormancy and the exact mechanism of transition to overt metastases is unclear. Likely factors involved in this transition include host microenvironment, host immune response, and genetic changes in the tumor cell.

3. Phenotypic Variability between CTC and Primary Tumor

Several studies have found genotypic variation between primary tumor and CTCs/DTCs of particular interest is the incongruent HER2 status between primary tumor and CTCs/DTCs. A recent study utilizing the CellSearch System in metastatic breast cancer found that 29% of HER2-negative primary tumors had HER2-positive CTCs and 42% of HER2-positive primary tumors had HER2-negative CTCs [13]. Another study by Fehm et al. looked at serum HER2 and CTCs in initially HER2-negative or HER2-unknown metastatic breast cancer patients. Of the 77 patients, 23/77 patients were HER2 positive based on either CTC detection or peripheral blood ELISA. HER2 concordance between CTCs and serum HER2 was 71%. HER2 status of the metastatic tissue was assessed in ten of these patients in which 2/10 had discordance between primary tumor and metastatic site [14]. Similar discrepancies have been reported in other studies, mostly ranging from 7 to 40%, as well as intermetastatic site variability [14–19]. Discordant HER2 status between primary tumor and CTCs/DTCs has also been reported in early breast cancer [20–23].

There has been conflicting evidence for treatment based upon CTC/DTC HER2 status. In another study by Meng et al., 9/24 patients initially HER2 negative acquired HER2 gene amplification throughout their disease process. These patients were either far advanced or had undergone previous chemotherapy or radiation. Four of the patients were treated with trastuzumab leading to one complete response and two partial responses [24]. Another study looked at trastuzumab response in 30 breast cancer patients stages 1–4 who had already completed standard therapy. All 30 of these patients had CK-19 mRNA-positive circulating and/or disseminated tumors cells present. Though only 33% of the primary tumors were HER2 positive, 83% of the CTCs and/or DTCs were HER2 positive. After trastuzumab therapy, 28/30 (93%) patients showed no CK-19 mRNA signal [25]. Similar results were reported from the National Surgical Adjuvant Breast and Bowel Project (NSABP) protocol B-31 suggesting a potential benefit from trastuzumab to HER2-negative patients [26, 27]. However, a large randomized phase 3 trial looked at randomization of trastuzumab with paclitaxel to metastatic breast cancer patients with HER2-negative primary tumors. Trastuzumab did not affect overall survival, response rate, or time to progression in non-HER2-overexpressing tumors [27]. A large European multicenter study (www.detect-study.de) is currently underway looking at CTC HER2 expression in metastatic breast cancer patients with HER2-negative primary tumors. This trial will look at several different techniques for determining HER2 status. It will also look at HER2-positive CTC response to HER2-targeted therapy [28].

4. CTC Detection Methods

If CTCs are to be used as surrogates for DTCs, then accurate and reproducible techniques are needed for CTC quantification. This is especially important when considering that CTC concentration in peripheral blood can be as low as one per 10^5–10^7 cells [29]. CTC detection occurs in a two-step process, enrichment, and identification. Several techniques are available for CTC enrichment. The Ficoll and OncoQuick systems utilize a density gradient centrifugation. These systems lack specificity as they separate CTCs and mononuclear cells from red blood cells and granulocytes [29]. Furthermore, CTCs can migrate between layers and the layers themselves can lose their integrity. Between the two systems, Gertler et al. found the OncoQuick system to be superior due its ability to select out mononuclear cells [30]. The ISET (Isolation by Size of Epithelial Tumor Cells) technique implores a filter consisting of 8 μm pores to separate CTCs from leukocytes [31]. However, small CTCs can be lost, and large leukocytes can be retained by the filters leading to poor sensitivity and specificity [29]. In general, these techniques have fallen out of favor, and most researchers use immunological techniques for enrichment.

Immunomagnetic systems target an antigen with an antibody that is coupled to a magnetic bead. They then isolate the antigen-antibody complex via exposure to a magnetic field. Enrichment can occur through either positive selection where the antibody is targeted against a CTC antigen (CKs, EpCAM, HER2) or negative selection where the antibody is targeted against a leukocyte antigen (CD 45 or 61). To date, the only FDA-approved system is CellSearch,

an immunomagnetic system that uses anti-EpCAM and anti-CD45 antibodies. The main limitation is heterogeneity of CTCs to express EpCAM. Furthermore, EpCAM is downregulated in malignant cells though a process called epithelial to-mesenchymal transition. Despite this, the CellSearch System has a high reproducibility rate [1, 32].

Once enrichment is completed, characterization of CTCs is achieved through molecular or immunological techniques. RT-PCR methodologies target tumor-specific antigens. This technique was initially considered to be sensitive; however, other authors have found several limitations to this technique [33, 34]. These limitations included false positives due to illegitimate gene transcription, contamination by pseudogenes, and transcription of markers present on nonmalignant cells [35, 36]. Furthermore, false negatives may arise if CTCs do not express the gene of interest [29]. Lastly, RT-PCR relies on cellular lysis which precludes further CTC analysis and assessment of CTC quantity. However, more recent developments in techniques allow for increased sensitivity and specificity by overcoming these pitfalls. Multimarker RT-PCR and novel primer designs avoid false positives [37]. In addition, advances in PCR technology with quantitative real-time RT-PCR (RT-qPCR) allows for cutoff values of transcripts between cancerous and noncancerous cells, thereby designating what values are tumor-cell derived [38, 39].

There are several immunological techniques used for the identification process. As described earlier, the CellSearch System uses immunomagnetic technology for enrichment. In the identification stage, these cells are fluorescently stained for cytokeratins (CK8, 18, 19), common leukocyte antigen (CD 45), and a nuclear dye (4,6-diamino-2-phenylindole (DAPI)). A fluorescent microscope then detects and identifies CTCs as those cells that are CK+/CD45−/DAPI+. Fiber-optic array scanning technology (FAST) also utilizes fluorescent anticytokeratin antibodies as well as DAPI counterstain. Stained cells are then exposed to laser-printing optics that excite 300,000 cells/second. This affords similar sensitivity and specificity to conventional automated digital microscopy with a 500-fold increase in speed [40]. Laser Scanning Cytometry (LSC) rapidly scans and relocates multimarker immune-labeled cells for visual examination to separate viable from nonviable cells [41].

Multiparameter flow cytometry has been utilized by several authors for the detection of CTCs since multiple surfaces markers and DNA ploidy can be evaluated [42–44]. As such, flow cytometry affords a high specificity and in one study demonstrated a higher specificity than RT-PCR [45]. More recently, microchip technology has been described for detection of CTCs. The "CTC-chip" uses a microfluid platform by which CTCs in whole blood samples that interact with microposts coated in anti-EpCAM antibody [46]. The authors demonstrated a sensitivity of 99% in a cohort of patients with metastatic cancers. Epithelial immunospot (EPISPOT) is a technique based on the enzyme-linked immunospot assay. EPISPOT detects only viable tumor cells as evidence by their ability to secrete proteins (CK-19, MUC-1) in short-term cell cultures [47].

5. Metastatic Breast Cancer

Most of the literature thus far has examined CTCs in metastatic breast cancer. Cristofanilli et al. looked at 177 metastatic breast cancer patients in a multicenter prospective trial using the CellSearch System and found that the presence of CTCs before initiation of therapy was a predictor of both decreased progression-free survival as well as overall survival. Through stratification according to progression-free survival, a cutoff value of 5 CTCs per 7.5 ml of blood was used to distinguish patients with a favorable versus unfavorable prognosis [48]. Several subsequent studies found similar conclusions [49, 50], and further followup data revealed elevated CTC counts at any point during therapy was associated with decreased progression-free and overall survival [15, 51, 52]. The prognostic value of CTCs has been shown to be superior to tumor burden, disease phenotype, and current imaging methodologies [53, 54]. CTCs also allow for molecular profiling. Gradilone et al. looked at CTC chemoresistance profiles in metastatic breast cancer patients using RT-PCR to quantify the number of multidrug-resistance-related proteins (MRPs) expressed. Those patients with greater than two MRPs expressed per CTC had a shorter progression-free survival then those with more drug-sensitive CTC profiles [17]. The next step in CTC research is currently being undertaken by the Southwestern Oncology Group (SWOG: S0500) via a phase three randomized trial looking at changing therapy versus maintaining therapy in metastatic breast cancer patients who have elevated CTC levels after the first followup visit upon treatment initiation.

Lastly, it has been recently shown in a mouse model for metastatic breast cancer that CTCs can also colonize their tumor of origin. This work completed by Kim et al. has been referred to as "tumor self-seeding." In their experimental model, the primary tumor was able to be seeded by separate tumor masses, metastatic lesions, and from direct inoculation. They found that the primary tumor secretes several cytokines that attract the CTCs, such as IL-6 and IL-8. In turn, once the CTCs have infiltrated the tumor, they secrete factors that influence the primary tumor microenvironment, including tumor growth, angiogenesis, and leukocyte recruitment. Thus, once further elucidated, the factors involved in CTC-primary tumor interactions allow for potential therapeutic targets [55].

6. Early Breast Cancer

There have been few studies regarding CTCs and early breast cancer. The reported CTC positivity rate has ranged from 9.4 to 48.6% [20, 22, 23, 56–67]. Several of these studies have tried to identify primary tumor characteristics that would predict the presence of CTCs. A recent study by Krishnamurthy et al. looked at DTCs and CTCs in stage 1 and 2 breast cancer patients and found that the presence of both DTCs and CTCs was independent of lymph node status, tumor grade, tumor size, and receptor status [64]. This is in contrast with early findings of the SUCCESS trial. This trial is looking at CTCs at the time of primary diagnosis as well as during adjuvant chemotherapy. They report a positive

correlation between lymph nodes status and CTCs [68]. Lang et al. looked at both CTCs and DTCs and found that only HER2 status of the primary tumor was associated with the presence of CTCs [62]. In contrast to previous studies, both Krishnamurthy et al. and Lang et al. did not find a correlation between the presence of CTCs with DTCs [62].

Several studies involving early breast cancer patients have shown that the presence of CTCs is associated with a worse prognosis. Wulfing et al. used a buoyant density gradient and immunomagnetic separation technique to look at HER2-positive CTCs in stage 1 through stage 3 breast cancer patients. They found that 17/35 (48.6%) patients had HER2-positive CTCs, of which twelve of these patients had HER2-negative primary tumors. The presence of HER2-positive CTCs was associated with a significantly decreased disease-free survival and overall survival [65]. This was validated by a recent large study of 216 patients using an RT-PCR technique to look at HER2-mRNA-positive CTCs [23].

Other studies have looked at RT-PCR techniques using mammaglobin A and CK-19. Ignatiadis et al. used a triple primer RT-PCR technique using CK19, mammaglobin A, and HER2 in 175 women with early breast cancer. They found that the presence of CK-19 mRNA-positive and mammaglobin A mRNA-positive CTCs prior to the initiation of adjuvant therapy was associated with a decreased disease-free survival [59]. However, a previous study by Ignatiadis et al. looking at 444 early stage breast cancer patients found that the presence of CK-19 mRNA-positive CTCs was associated with a reduced disease-free survival in only ER-negative, triple-negative, and HER2-positive patients [60]. Xenidis et al. looked at 167 node-negative breast cancer patients and found that the presence of CK-19 mRNA-positive CTCs was associated with both early clinical relapse and disease-related death [58]. Ntoulia et al. and Ferro et al. found that mammaglobin A mRNA positivity was associated with a poorer prognosis [61, 67].

7. Treatment Monitoring

One goal of CTC detection is to be correlate CTC levels to disease progression and response to treatment. In early breast cancer, some studies have found a correlation between initial CTC reduction upon therapy initiation and the final response of the tumor [69, 70]. However, most studies in early breast cancer do not support a correlation between CTC response and tumor response. The GeparQuattro study looked at CTC levels at the time of diagnosis and after neoadjuvant therapy in 213 large operable and locally advanced breast cancer patients. Neoadjuvant therapy included trastuzumab if the primary tumor was HER2 positive. The incidence of CTCs went from 21.6% before treatment to 10.6% after treatment. Fifteen percent of initially CTC-positive patients became CTC negative after treatment, and 8.3% of initially CTC-negative patients became CTC-positive after treatment. However, no significant correlation was found between CTC detection and the primary tumor's response to neoadjuvant therapy [20]. Pierga et al. found similar results in a study of 118 stage 2-3 breast cancer patients using the CellSearch System. Though 23/118 (19%) patients had a complete response to neoadjuvant therapy, changes in CTC count did not correlate with tumor response [66].

Though data is inconsistent regarding tumor response, most studies have found that the presence of CTCs does predict early relapse. The SUCCESS trial looked at 1,489 nonmetastatic breast cancer patients using the CellSearch System and found that pretreatment CTC detection was associated with reduced disease-free survival as well as overall survival, while post treatment CTC detection was only associated with reduced disease-free survival [68]. Similar results were shown in the previously described study by Pierga et al. [66]. The data from Pierga et al.'s study was further analyzed after a longer followup period (18 months versus 36.4 months) and concluded that preneoadjuvant CTC detection is a better predictor of overall survival and distant metastasis-free survival than post-treatment CTC detection [56]. Xenidis et al. looked at 437 early stage breast cancer patients and found both pre- and post-treatment CK-19 mRNA-positive CTCs to be associated with decreased disease-free and overall survival [58]. Apostolaki et al. looked at HER2 mRNA-positive CTCs in 214 stage 1 and 2 breast cancer patients. Initially HER2 mRNA positivity was 21%. Adjuvant chemotherapy was able to eliminate CTCs in 16/53 (30.2%) of patients. The presence of CTCs after treatment was associated with reduced disease-free interval [22]. Similar results were reported in a study looking at adjuvant therapy which found that an increase in CTC level of tenfold or higher, independent of an initial response, predicted early relapse [71].

Several studies have looked specifically at CTCs during treatment with hormonally therapy. Pachmann et al. recently found that escalating numbers of CTCs during Tamoxifen treatment was a strong predictor of relapse. This increase was also predictive of subsequent relapse during aromatase inhibitor treatment [72]. Furthermore, Xenidis et al. reported a reduced disease-free interval as well as overall survival with persistent CK-19 mRNA CTC positivity after treatment with Tamoxifen [73].

8. Conclusion and Future Directions

Evidence has shown that CTCs play a prognostic role in both early and metastatic breast cancer patients. In early breast cancer, the presence of CTCs allows clinicians to identify those patients that are at risk for recurrence and therefore may benefit from additional therapy. In both early and metastatic breast cancer patients, CTCs are an easily assessable source for monitoring treatment efficacy. Though results from the SWOG trial are pending, CTC monitoring may allow oncologists to change therapy earlier in disease progression. Lastly, with molecular and genetic characterization of CTCs, chemoresistance profiles should also be able to advise the clinician of the most efficacious chemotherapy regimens.

In terms of tumor biology, it is clear that circulating tumor cells are present in early breast cancer thus supporting

the theory of early metastasis. One question yet to be answered is exactly how early in the neoplastic process does tumor dissemination occur. Studies have yet to look at the presence of CTCs in ductal carcinoma in situ (DCIS). Not all CTCs may lead to metastatic deposits as the metastatic niche may need to be created. Furthermore, even in early breast cancer, CTCs show great diversity compared to the primary tumor. Of particular interest is the diversity in HER2 status. It may be possible to target CTC/DTC to eradicate potential metastatic deposits. Targeting CTC using vaccines against HER2 and other pathways involved with breast cancer could theoretically decrease the probability of CTC seeding, recurrence, and/or metastasis [74–77].

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Review Article

Blood-Brain Barrier Integrity and Breast Cancer Metastasis to the Brain

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Brain metastasis, an important cause of cancer morbidity and mortality, occurs in at least 30% of patients with breast cancer. A key event of brain metastasis is the migration of cancer cells through the blood-brain barrier (BBB). Although preventing brain metastasis is immensely important for survival, very little is known about the early stage of transmigration and the molecular mechanisms of breast tumor cells penetrating the BBB. The brain endothelium plays an important role in brain metastasis, although the mechanisms are not clear. Brain Microvascular Endothelial Cells (BMECs) are the major cellular constituent of the BBB. BMECs are joined together by intercellular tight junctions (TJs) that are responsible for acquisition of highly selective permeability. Failure of the BBB is a critical event in the development and progression of several diseases that affect the CNS, including brain tumor metastasis development. Here, we have delineated the mechanisms of BBB impairment and breast cancer metastasis to the brain. Understanding the molecular mediators that cause changes in the BBB should lead to better strategies for effective treatment modalities targeted to inhibition of brain tumors.

1. Introduction

Breast cancer patients often develop metastatic lesions in the brain [1, 2]. The development of CNS metastasis in patients with solid malignancies represents a turning point in the disease process. The prevalence of CNS metastasis from breast cancer may be increasing due to improved systemic therapy for stage IV breast cancer. The standard treatment for multiple brain lesions remains whole-brain radiation for symptom control, with no improvement in survival. The therapy for a single brain metastasis remains either surgery or radiosurgery, with conflicting information as to the benefit of prior whole-brain radiation.

To metastasize to the brain, breast cancer cells must attach to microvessel endothelial cells and then invade the blood-brain barrier (BBB), which constitutes the endothelium and the surrounding cells. The BBB is a unique anatomical structure that is mainly defined by tight junctions and adherens junctions between the brain endothelial cells, that strictly regulate the flow of ions, nutrients, and cells into the brain [3, 4]. Compared with endothelial cells from other vascular beds, brain microvascular endothelial cells (BMECs) characteristically have very low permeability to solutes, high electrical resistance, complex tight junctions, and an array of transport systems that both supply the brain with nutrients and eliminates byproducts of brain metabolism. The low permeability is also important in protecting the brain from toxins circulating in the blood and restricting the migration of leukocytes and monocytes. The BMECs form an active permeability barrier and transport system known as the BBB, which is instrumental in the control of the brain fluid milieu. A widely supported hypothesis is that tumor cell adhesion to endothelium induces a retraction of the endothelium, which exposes the vascular basement membrane to the tumor cells. Numerous studies have shown that tumor cells recognize and bind to components in the vascular membrane, thereby

initiating extravasation and the beginning of new growth at secondary organ sites. The impairment of the BBB was observed recently in breast cancer patients who developed metastasis to the brain [5].

The BBB, a regulated interface between the peripheral circulation and the central nervous system (CNS), is comprised of the cerebral microvascular endothelium, which together with neurons, astrocytes, pericytes, and the extracellular matrix, constitute a "neurovascular unit" (Figure 1) [3, 4, 6]. The BBB is a highly selective diffusion barrier at the level of the cerebral microvascular endothelium, characterized by the presence of mainly tight cell-cell junctions, adherens junctions and lack of fenestrations (Figure 2). The BBB regulates bidirectional control over the passage of a large diversity of regulatory proteins, nutrients and electrolytes, as well as potential neurotoxins [7, 8].

Increased BBB permeability can be either a consequence of the pathology or a precipitating event [7, 8]. Impairment of the BBB leads to an increase in permeability and formation of edema. Inflammatory mediators such as histamine, bradykinin, and Substance P cause increase in permeability of BBB *in vivo*, which results from the rapid formation of endothelial gaps [7, 8].

2. Tight Junctions and Blood-Brain Barrier Integrity

Most forms of brain injury are associated with BBB disruption, resulting in secondary damage to neural cells. The interendothelial space of the cerebral microvasculature is characterized by the presence of a junctional complex that includes adherens junctions (AJs), tight junctions (TJs) and Gap junctions [8] (see Figure 3). Whereas gap junctions mediate intercellular communication, both AJs and TJs act to restrict the permeability across the endothelium. AJs are ubiquitous in the vasculature and mediate the adhesion of endothelial cells to each other, contact inhibition during vascular growth and remodeling, initiation of cell polarity and partly the regulation of paracellular permeability. The primary component of AJs is VE-cadherin. The *TJs* are the main components that confer the low paracellular permeability and high electrical resistance. TJs are elaborate structures that span the apical region of the intercellular cleft of endothelial barrier tissues. TJs function both as a "zipper" and a "fence" that limit paracellular permeability and are composed of transmembrane proteins as primary seals linked via accessory proteins to the actin cytoskeleton. The TJs are composed of a complex of belt-like zonula occludin, which is localized close to the lumen of the capillary. Electrical resistance *in vivo* across the barrier can increase to approximately 1200 ohm·cm^2 or higher due to the TJs. The proteins of the TJs include the junctional adhesion molecules (JAM) (JAM-1, JAM-2 and JAM-3), occludin, the claudins, and zonula occludin proteins (ZO-1 and ZO-2). Interestingly, brain microvascular endothelial cells do not express ZO-3 [8].

The ZO proteins are involved in the coordination and clustering of protein complexes to the cell membrane and in the establishment of specialized domains within the membrane [3]. ZO-1 links transmembrane proteins of the TJ to the actin cytoskeleton. The primary cytoskeletal protein, actin, has known binding sites on all ZO proteins and on claudins and occludin. Actin filaments serve both structural and dynamic roles in the cell. ZO-1 binds to actin filaments and to the C-terminus of occludin and claudins, which couples the structural and dynamic properties of perijunctional actin to the paracellular barrier.

The numerous pathways by which specific TJ proteins are regulated and the specific effects of certain pathologies on tight junction (TJ) proteins strongly suggest that therapies targeted to components of the TJ complex and its modulators for the treatment and prevention of breast metastasis to the brain and development of brain tumors are a promising avenue that needs to be explored.

3. Genes That Mediate Breast Cancer Metastasis to the Brain

The molecular mediators that influence metastasis in distant sites appear to vary by organ (Figure 4). In malignancies of the breast, cancer cells enter a prolonged period of latency before they gain competence to colonize and produce organ-specific metastases [10–12]. During this period of time, disseminated cancer cells may acquire distinct sets of metastasis functions depending on the target organ [13, 14]. Despite the various infiltration and colonization functions, the general process of metastasis can be broken down into local invasion, intravasation, survival in the circulation, extravasation and colonization [15] (Figure 5). After intravasation the cancer cells need to survive in the circulation, travel to specific target organs and extravasate into a microenvironment where they can colonize as secondary tumors [15]. Searches for genetic determinants of metastasis have led to identification of gene signatures that selectively mediate breast cancer cell metastasis to bones, the lungs, and the brain [13–15]. Based on previous work on genomic analysis of breast cancer metastasis to bone and lung, the Massagué group identified three tumor metastasis genes that mediate extravasation through the BBB and cancer cell colonization in the brain [15]. The barriers to metastasis are distinct in organs. To colonize the brain parenchyma, invading tumor cells must penetrate the blood-brain barrier (BBB). Brain capillary walls are more difficult to penetrate due to a tight layer of endothelial cells, tight junctions, and astrocyte foot processes [10, 16]. Functional validation of these genes provided clues as to how cancer cells can penetrate the BBB and initiate tumor growth in brain vasculature. A brain metastasis signature (BrMS) consisting of 17 genes was created using genomic profiling and univariate analysis. The cycloxygenase-2 (COX2), the epidermal growth factor receptor (EGFR) ligand HB-EGF, and the α2, 6-sialyltransferase (ST6GALNAC5) were identified as mediators in cancer cell extravasation and infiltration through the BBB. The expression of COX-2 and EGFR ligand HB-EGF enhances the extravasation of cancer cells across the capillaries in an *in vivo* animal model system. The ST6GALNAC5 expression is restricted to the brain both in mice and humans [17]. The knockdown of ST6GALNAC5 reduced the cell passage

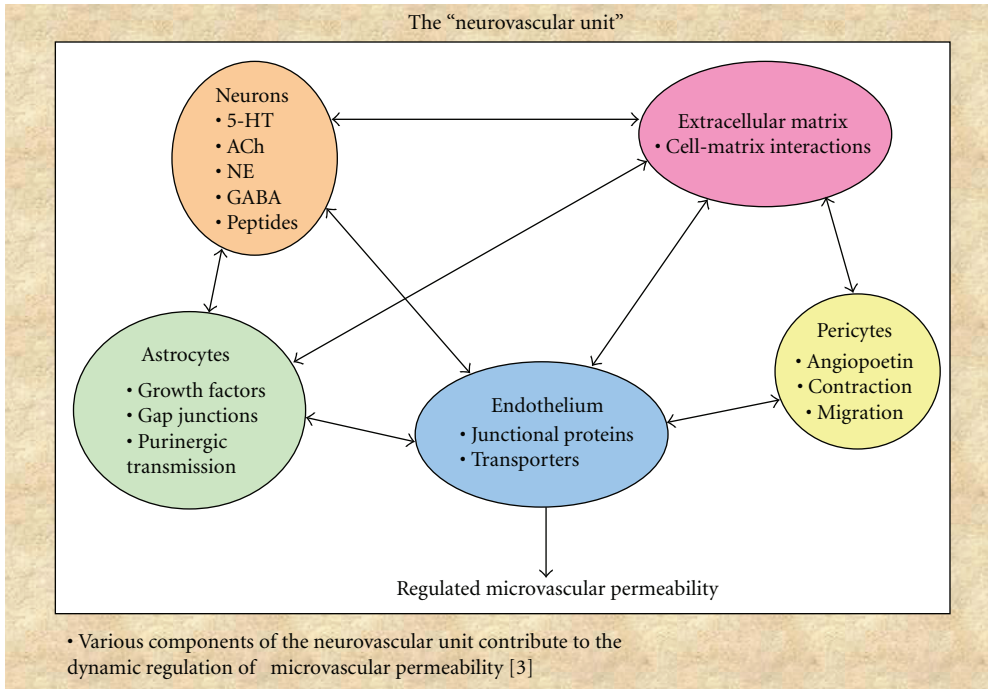

Figure 1

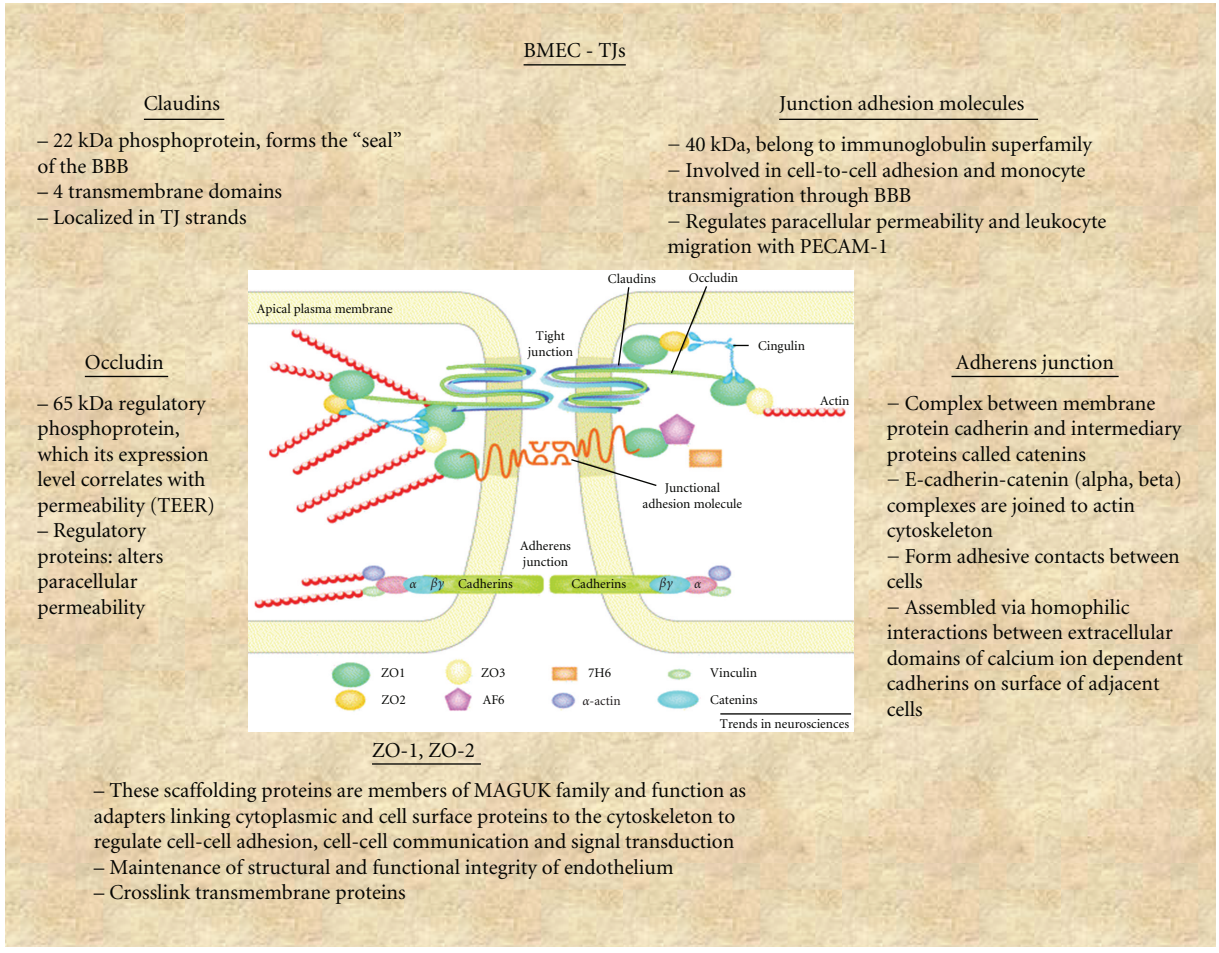

Figure 2: Schematic Presentation of TJs Structures in BMECs [9].

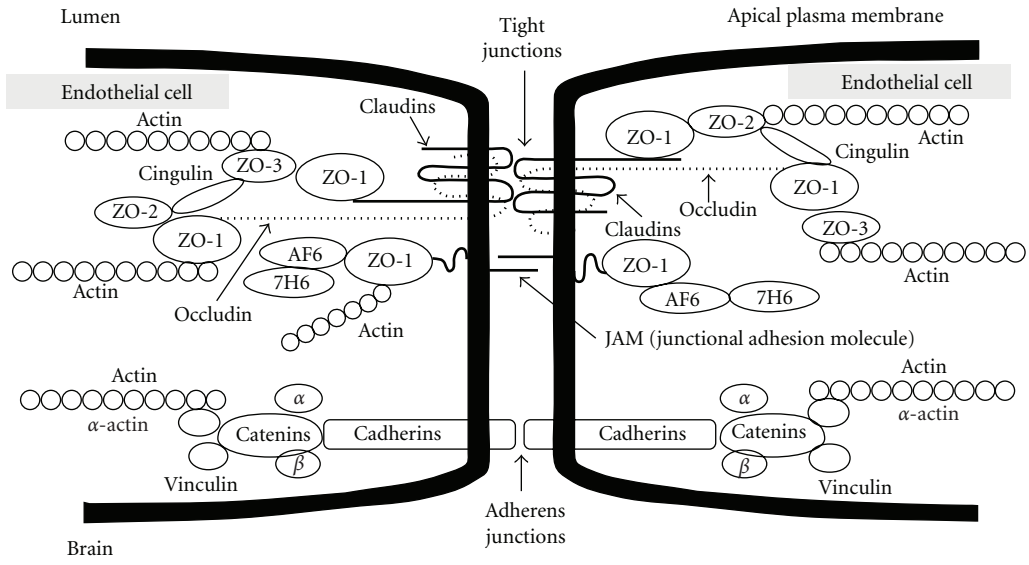

Figure 3: Proposed molecular organization of blood-brain Barrier tight junctions [9].

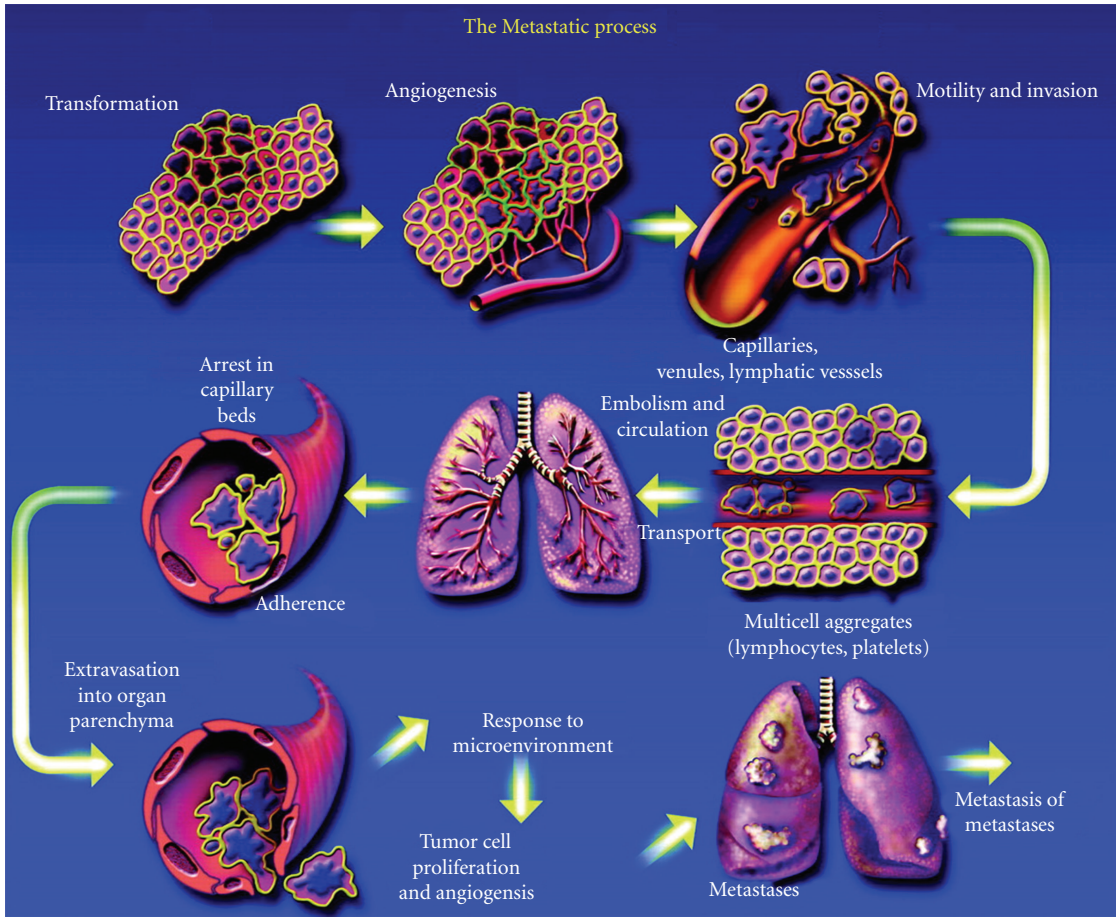

Figure 4: Cancer metastasis. Pathogenesis of cancer metastasis: the process of cancer metastasis consists of sequential, interlinked, and selective steps. The outcome of each step is influenced by the interaction of metastatic cells with homeostatic factors. Each step of the metastatic process is considered rate limiting in that failure of a tumor cell to complete any step effectively terminates the process. Therefore, the formation of clinically relevant metastases represents the survival and growth of unique subpopulations of cells that preexist in primary tumors.

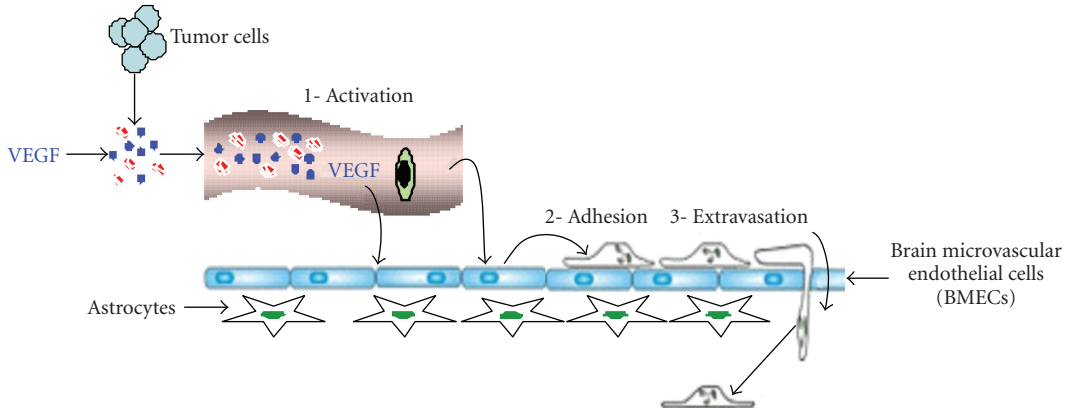

Figure 5: Schematic presentation of tumor cell penetration across the BBB.

through a BBB and suppressed metastasis to the brain. In an *in vitro* model of BBB, which consisted of human primary endothelial cells and astrocytes, Massagué and colleagues demonstrated that the ST6GALNAC5 can increase cancer cell adhesion to brain endothelial cells and infiltration through the BBB. The Massagué group has previously identified four lung metastasis gene signature (LMS) that contribute to vascular remodling of tumor blood vessels, entry into the circulation and passage into the lung parenchyma [13]. Comparison of the BrMS with the lung metastasis signature (LMS) showed an overlap of genes between signatures, but not in the bones or liver. Some of the overlapped genes include COX-2, EGFR ligand, ANGPTL4, and LTBP1, which are known to promote disruption of the endothelial barrier and metastasis to the brain and lung. The Massagué group suggested that these genes may specifically contribute to expression signatures that are predictive of metastasis in the brain.

4. Cooption of Tumor Cells with Brain Endothelium

Given the observations that certain cancers may have preferential metastatic sites it is natural to investigate what factors, if any, make the brain an "attractive" target for tumor cell growth; specifically in regards to breast primary tumors. The widely accepted "seed and soil" hypothesis first offered by Piaget in 1889 has been credited as the most plausible explanation for the targeted behavior seen in the progression of cancer growths [18]. If accepted, it follows that brain tissue (the "soil") consisting of neurons, extensive vasculature, and associated neuropil have trophic effects that attract breast primary tumor cells (the "seed") and facilitate their growth.

To deduce the validity of this, still prevalent, century-old hypothesis it is vital to observe metastasis before, during, and immediately following successful "colonization" of the distant site. It is within this time frame that any mechanisms, such as Paget's proposal of trophic factors, may play a central role. The time point of interest, referencing current knowledge of metastatic progression, lies in the events between extravasation into distant tissue and any subsequent neoangiogenesis-driven growth (Figure 4) [19].

It is important to emphasize at this time that the current discussion will focus on the breast-brain relationship. The genetic heterogeneity of migrating tumor cells is well documented and undoubtedly contributes to profound differences in interaction involving other tissues and organs [20, 21]. Carbonell and colleagues are equally cautious of this distinction, especially in light of their data and its contradiction to the Piagetian "soil" concept. In their paper they reference the relative lack of direct evidence for Paget's hypothesis based on *in vivo* studies [22]. It is this lack of convincing proof that prompted the study of breast cancer cell migration to the brain with a greater focus on the specific steps that lead to successful colonization.

In their paper, direct observation of early tumor colonization revealed a predisposition for growth around existing brain vasculature. This vascular "cooption" contradicts the notion proposed by Paget that trophic factors from distant tissue are responsible for the initial establishment of migrating tumor cells. This is not to deny the possibility that cytokines and chemokines are responsible for drawing tumor cells to certain areas as they traverse the systemic circulation. The possibility of chemoattraction via the CCR7 and CXCR4 receptors has been shown and recognized [23]. Upon arrival, tumor cells preferentially attach to existing blood vessels [22] rather than the chemoattractant releasing neural tissue as expected from the Piagetian viewpoint. Thus, from current knowledge it can be inferred that trophic signaling could play a role in the macroscopic targeting of breast primary tumor cells to the brain, but that the same factors may have a diminished role once access to brain tissue has been attained. They based their initial experiments on the behavior of the MDA-MB-231 cell line. Interestingly, they conducted identical tests with the "brain seeking" MDA231BR cell line as well as A7 (human melanoma) and K1735M2 (murine melanoma) cell lines. All cell lines tested exhibited behaviors consistent with vascular cooption.

The underlying similarity between all conditions and tests is the brain host tissue, its vascular basement membrane and HBMECs. The tight junctions and associated pericytes of the blood brain barrier are a difficult challenge for any invader to penetrate. This includes "invasion" by researchers and clinicians attempting to deliver chemotherapeutic agents

and other drugs [24]. The slower rate of extravasation in brain is well noted in comparison to the fenestrated capillaries of other tissues such as bone and liver [25]. It is this unique property of brain microvasculature, a tightly regulated series of junctional complexes that may explain the "antiPiagetian" findings described above. The question becomes whether or not this difficulty in extravasation directly promotes the viability of vascular cooption over direct attachment and growth on neural tissue. It is important to note that these recent findings on vascular cooption within the brain do not diminish the substantial effect of neoangiogenesis on subsequent growth in tumor size and scope. It has been shown that lack of new blood vessel formation and/or remodeling often leads to the death or incapacitation of tumorigenic tissue [26]. Successful migration and initial attachment are steps that must be conceptually separated from the unregulated macroscopic growth that commonly defines cancer. Thus, vascular cooption [22] is the most reliable method by which breast primary tumor cells are able to procure the necessary nutrients and physical scaffolding for initial implantation and growth within the brain.

5. Colonization of Tumor Cells around the Blood Vessels

The importance of vascular cooption as a means for tumor cells to survive is highlighted in a study by Gevertz and Torquato [27]. They explain that neoplastic growth is possible even with angiogenesis inhibited as long as vascular cooption is an alternative [27]. Nonetheless, they also report that neoangiogenesis and vascular remodeling is necessary if tumor masses are to grow beyond 1-2mm in diameter. As aforementioned migration and early attachment/colonization should be considered separate from the macroscopic growth step. Clearly, it is the proximity to, as well as early and ongoing interaction with blood vessels in the brain, that contributes significantly to tumor cell fate.

The focus of Gevertz and Torquato on the effects of VEGF, Ang-1, and Ang-2 are interesting in their interplay. They find a pattern of vascular cooption, vessel regression, and robust angiogenesis that requires tight regulation of these factors [27]. The possibility of regulation at the gene level warrants further study. Such a mechanism supports data on the genetic heterogeneity of primary tumor cells and the Darwinian selection of those tumor cells with the capability for metastasis [28].

It is known that primary tumors can shed more than a million cells per gram of the tumor mass a day [19]. Despite this constant dispersal of tumor cells, and despite public fear and opinion, metastasis is relatively difficult and inefficient. Thus, the study of physiological changes due to changes at the gene level is a promising direction for cancer research. In light of the importance of vascular cooption and blood vessel colonization to invading tumor cells, a look at gene-regulated factors influencing vascular cooption and colonization could provide a clue to the prevention of secondary growths.

angiogenesis, as we have discussed, is a late event when considering first the chemotaxis of tumor cells, extravasation past the blood brain barrier, and finally successful vascular cooption. The steps preceding angiogenesis, according to Lorger and Felding-Habermann contribute to the lower success rate of brain metastasis compared with other tissues [25]. They report that tumor cells extravasating into brain parenchyma were found to be arrested in G_0 of the cell cycle. These findings suggest an amount of stress and energy expenditure consistent with a greater effort needed in penetrating the intercellular junctions already discussed in this paper. It is well known that loss of cell attachment proteins and mechanisms leads to the shedding of material from primary tumors [20]. The loss of function in E-cadherin through the disruption of alpha-catenin and/or beta-catenin is well known [1]. We have revealed here that the process for metastasis could very well complete the circle, at least in regards to breast-brain metastasis. Just as loss of adhesion is a necessary first step for tumor cells to leave their primary tissue site, prompt adhesion to the vascular basement membrane of brain endothelial cells is required (and sufficient) for initiation of secondary growth. From evidence collected thus far, it is a possibility that attachment proteins and their constituents largely assume control of primary tumor cell fate as soon as extravasation into brain tissue is complete; wresting control away from any trophic factors. There is evidence that the presence of the blood brain barrier would make such a shift in cellular interaction necessary. Regardless, early colonization around the brain's existing vasculature appears to be necessary for successful metastasis and has the potential for future clinical therapies aimed at prevention of secondary growths within the brain.

6. Reactive Astrocytes and Glia on Tumor Growth

The brain provides a unique microenvironment due to its distinctive structure of extracellular matrix (Table 1) and the blood brain barrier (BBB) [29]. It is known that interactions of the host microenvironment and metastatic cells affect the outcome of metastatic progression and tumor survival [30]. Lorger and Felding-Habermann provided in depth *in vivo* analyses of early changes in brain microenvironment upon arrival of breast cancer cells [31]. For studies of the breast cancer cell arrest and extravasation into the brain parenchyma, the Habermann group established breast cancer cell models using MDA-MB-231/brain cells, MDA-MB-435 and murine 4T1 cancer cells. After cell injection into left carotid artery of mice, astrocyte activation was detected in the left hemisphere in brain, showing consistent upregulation in the vicinity of intravascular arrested cancer cells. Reactive astrocytes surrounded and infiltrated brain metastases. Consistent astrocyte activation was detected throughout the extravasation process as well as upregulation of matrix metalloproteinase-9 (MMP-9) proteins in close proximity of extravasating cancer cells. The astrocytic MMP-9 factor can influence cancer cell invasion by promoting growth and angiogenesis in primary

Table 1

ECM molecules	Candidate or demonstrated receptors	Collagen	Integrins (a1fl1, a2fl1, a3/.3), CD44, syndecan, proteoglycans
Laminins	Integrins (a13, a2fl, a3f3, a6fl, and a7j3; avjIs, a6fl4), dystroglycan, lactose-binding lectins, proteoglycans	Thrombospondins	Integrins avfls, avj3x, axfij, CD36, syndecan, proteoglycans, sulfatides
		Tenascin	Fl 1, integrins (axflj) syndecan, cytotactin binding proteoglycans
Fibronectin	Integrins (av/33, avf36, asfli, a5fl), CD44, syndecan, proteoglycans	Proteoglycans	Hyaluronan, integrins

brain tumors through release of vascular endothelial growth factors (VEGFs) from the extracellular matrix [32]. In addition to angiogenesis, VEGF also has the function to support the survival and dissemination of breast carcinoma cells [33]. Habermann and Lorger suggested that early involvement of reactive astrocytes may influence the tumor cell fate within the brain parenchyma. In their study, some reactive astrocytes expressed nestin during early cancer cell invasion. In melanoma cells, astrocytes secret haparanase to support the brain microenvironment and the growth of metastatic cells [34], in addition to astrocytes, microglia responses to invading breast cancer cells were detected. Unlike astrocytes, microglia activation associated with the cancer cell brain colonization was not consistent. The active and reactive microglial populations displayed different phagocytic activities and morphology. Despite the differences, a variety of glial responses adds uniqueness to local brain microenvironment of which is essential in determining tumor cell invasion and progression. Astrocytes may have multiple functions in the brain microenvironment. In response to brain injury, astrocytes are activated and recruited to form a glial scar in the site of injury [33]. They can protect neurons from injury induced apoptosis [35]. The Fidler group determined whether reactive astrocytes can also provide neuroprotective properties on protecting tumor cells from cytotoxicity induced by chemotherapeutic drugs. *In vitro* study demonstrated that activated astrocytes protect tumor cells from chemotherapeutic drugs through direct physical contacts.

Astrocytes play important roles in maintaining homeostasis in the brain by regulating nutrient transport, ion trafficking across the extracellular matrix (Table 1) as well as neuronal signaling. It has been shown that specific interactions between brain endothelium and astrocytes within neurovascular units (Figure 1) can influence BBB permeability under pathological conditions. Interactions between the brain endothelium, astrocytes, and neurons may also regulate blood-brain barrier (BBB) function [36]. Cancer cell progression and survival depend on interplay between local host cells and invading tumor cells. Although the specific functions of astrocytes and microglia in early metastatic invasion are yet to be determined, studying local host cells responses during tumor cells invasion may lead to better understanding of tumor microenvironment. Such information could lead to a new avenue of therapeutic targets for brain metastases.

7. Angiogenesis and Brain Tumor Growth

New blood vessel formation plays an important role in breast cancer growth, invasion, and metastasis. Tumor growth is preceded by the development of new blood vessels, which provide a pathway for metastasis and nutrients essential for growth. Vascular endothelial growth factor A (VEGF) is a key angiogenic mediator that stimulates endothelial cell proliferation and regulates vascular permeability [37, 38]. Highly proliferative tumors, such as those that are negative for the estrogen, progesterone, and Her2/neu receptors have enhanced angiogenesis that supports rapid growth and early metastasis; also expressing high levels of VEGF [39]. Thus, breast cancer patients that have tumor cells secreting high levels of VEGF may have a higher risk of developing breast cancer metastasis to the brain. VEGF also acts in concert with Angiopoietin2 to regulate vessel growth. In human cancers, increased expression of Ang2 in tumor cells is closely correlated to tumor cell progression, invasiveness, and metastasis [40, 41].

VEGF is essential for angiogenesis and BBB functioning. Our previous studies showed that VEGF upregulated ICAM-1 via phosphatidylinositol 3 OH-kinase/AKT/Nitric oxide pathway and modulated migration of HBMECs [42]. Using human cytokine cDNA array, we found that VEGF-induced significant increase in expression of monocyte chemoattractant protein-1, the chemokine receptor CXCR4 as well as IL-8 in HBMECs [43]. VEGF increased IL-8 production in HBMECs through activation of nuclear factor-κB via calcium and phosphatidylinositol 3-kinase pathways [44]. We also showed that VEGF secreted from breast cancer cells significantly increased the adhesion and penetration of breast cancer cells across the HBMECs monolayer, via changes of VE-cadherin which were inhibited by SU-1498 inhibitor for VEGFR-2 and calcium chelator. VEGF also regulated focal adhesion assembly in HBMECs through activation of FAK and RAFTK/Pyk2 [45]. These focal adhesions are complexes comprised of scaffolding and signaling proteins organized by adhesion to the extracellular matrix (ECM). Further, VEGF upregulated the expression of α6 integrin and increased the $\alpha 6\beta 1$ integrin expression in HBMECs which were important for VEGF induced adhesion and migration as well as in vivo angiogenesis and tumor angiogenesis [46].

VEGF and its cognate receptors are central to the regulation of angiogenesis in both physiological and pathological states. In cancer, local tumor hypoxia stimulates VEGF synthesis and VEGF levels are subsequently elevated in

breast cancer. VEGF expression levels correlates with poor prognosis. Blocking of the VEGF-VEGF receptors pathway is accepted as the first antiangiogenic therapy. However, since tumors often develop evasive resistance to this therapy, the development of new antiangiogenic approaches is required for successful antiangiogenic therapy. This can be achieved by better understanding of the receptors and pathways involved in vascular remodeling in brain. Angiopoietins and Tie2 receptor complex were shown to play a critical role in tumor angiogenesis; however their roles in brain BMECs remain elusive.

VEGF is the most important factor in the regulation of the development and differentiation of the vascular system. By acting as a capillary permeability enhancing agent, VEGF also affects the integrity of the BBB. As primary partners of VEGF, angiopoietins (Angs) also play a multiple critical role in vascular development. Angiopoietins are ligands for the Tie 2 receptors and have either agonistic (Ang-1 or Ang-4) or antagonistic (Ang2 and Ang-3) actions regulating vascular survival and expansion. Ang2 is a natural antagonist of Angiogenesis in different microenvironments. Concerted expression of VEGF and Ang2 resulted in increased microvessel density in solid tumors [40]. Ang2 also upregulated MMP-1 and MMP-9 in the presence of VEGF in vitro and MMP elaboration, which participates in the induction of microvessel sprouting in the growing vascular network.

8. Clinical Aspects of Breast Cancer Metastasis to the Brain

Brain metastasis, is a significant cause of morbidity and mortality in patients with breast cancer. HER-2 positivity is an increasing recognized risk factor for the development of brain metastasis [47]. Other than Her2 overexpression, there are other factors that increase the risk for breast cancer metastasis to the brain such as negative estrogen and progesterone receptor status, young age, large tumor size, elevated Lactate dehydrogenase (LDH), grading, and number of positive lymph nodes [48].

As breast cancer is the second most common cause of brain metastasis (after lung cancer) occurring in 10–15% of patients with breast cancer, autopsy studies suggest that the actual incidence is twice (~20 to ~30%) [47]. The incidence of brain metastases is thought to be increasing due to the introduction of more sensitive and accurate diagnostic methods and screening techniques. During the last decade, improved adjuvant and palliative therapy regimens have led to improvement in survival of these patients. In a majority of these patients, the central nervous system dissemination occurs several years (~5 to ~20 years) after systemic lesions have been diagnosed. Approximately 70–80% of the lesions are not solitary but multiple. Cerebrum is the most common site for breast cancer metastasis, following the cerebellum and brainstem [48].

Clinically, this parenchymal brain metastasis have an insidious onset with headache (24–48%), neurological deficits as focal motor weakness (16–40%), altered mental status and cognitive dysfunction (24–34%). Seizures, ataxia, nausea, vomiting can also be presenting symptoms. Leptomeningeal metastasis is presented with nonlocalizing symptoms such as headache, nuchal rigidity or cranial neuropathies.

Brain metastasis can be diagnosed through various techniques. Gadolinium-enhanced magnetic resonance imaging (MRI) is more sensitive than contrast enhanced computed tomography (CT) for identifying both Parenchymal and leptomeningeal disease and is therefore preferred method for detection of brain tumors. Contagious thin axial slices without skips are necessary to pick up small lesions that are missed on CT, especially, in the front-temporal region and in the posterior fossa and brainstem. MRI is also superior in differentiating between solitary and multiple lesions. Approximately 20% of patients thought to have single brain metastases on CT actually have multiple lesions on MRI. Stereotactic brain biopsy must be considered where diagnosis of metastasis is in doubt, especially in patients with a typical presentation as it would lead to change in diagnosis in about 11% of cases. Primary brain tumors, infections, infarction and radiation necrosis are the likely alternative possibilities.

Treatment of brain metastasis depends on many factors as such location, number of metastasis, age of the patient, performance, status, and localization of extra cerebral lesions and a prediction of their responses to systemic therapy. On the basis of all these findings, a clinician can decide to have either invasive or noninvasive treatments. Historically, the incidence of clinically appearing CNS metastases in patients with breast cancer is 10–20%. The median time from diagnosis of breast cancer to CNS metastases is about 33 months with 5 months median survival time once diagnosed with cerebral involvement [47]. The majority of cancer patients who develop metastatic brain disease, present with multiple lesions, and death are attributed to uncontrolled metastatic brain disease in approximately 40% of the patients. Median survival in untreated patients with CNS involvement is 1 month; in patients administered with corticosteroids, the survival rate can go to 2 months; and following CNS radiotherapy it can go to 3–6 months. Patients with single CNS lesions and limited systemic disease amenable to surgery or radiotherapy may achieve median survival in the range of 10–16 months.

As mentioned earlier, the treatments, prognosis, diagnostic criteria could be different for two types of metastasis, parenchymal and leptomeningeal. The management of patients with brain metastasis can be divided into two groups one for leptomeningeal and other for parenchymal metastasis. Further, there are two approaches for treatment one is symptomatic and the other definitive. Corticosteroids and Anticonvulsants are symptomatic treatments, while the definitive treatment includes whole-brain radiotherapy (WBRT), surgical resection, stereotactic radiotherapy (SRS), whole-brain radiotherapy with radiosensitizers, intracavitary and interstitial brain irradiation, chemotherapy and Chemoradiotherapy.

8.1. Leptomeningeal Metastasis. Breast cancer metastasis is the most common cause of metastasis to the leptomeninges,

especially from a lobular carcinoma [49]. As described earlier, the symptoms presented are headache, vomiting, ataxia, lethargy, spinal symptoms, cranial nerve palsies and very rarely seizures. Definitive diagnosis is by Cerebrospinal fluid analysis for the presence of malignant cells. Focal radiotherapy is given to symptomatic and bulky sites. The treatment of the entire neuraxis can lead to unacceptable toxicity, mainly leukoencephalopathy and dementia. Those, whose extracranial disease is reasonably controlled, intrathecal chemotherapy can be done through Ommaya reservoir or via lumbar puncture. The most commonly used chemotherapeutic drugs are methotrexate, thiotepa and more recently liposomal cytarabine (Depot Cyt) [50]. The median survival even after multimodality therapy is only 12 weeks.

8.2. Parenchymal Metastasis. The most common form of metastasis is thought to be spread via hematogenous route. The management and prevention of CNS metastasis in patients whose tumors over express HER-2/neu need to be reevaluated in the present trastuzumab era, with special consideration for prophylactic cranial irradiation, as trastuzumab is known to increase the incidence of brain metastasis in this group of patients [51–53]. Along with the effectiveness of stereotactic surgery and newer radiotherapy techniques, innovations in blood-brain barrier disruption have expanded the scope of less damaging systemic therapies in brain cancer including metastases [54].

8.3. Chemotherapy. The impermeability of BBB to ionized water soluble compounds >180 Da and the presence of the P-glycoprotein efflux pump at the luminal surface of the brain capillaries result in lack of penetration of the chemotherapeutic drugs. Though breast cancer is a chemosensitive disease, there is limited data on the use of chemotherapy for breast cancer metastatic to brain. Most commonly used are cyclophosphamide-based regimen (along with methotrexate, 5FU, prednisolone, etc.), producing response rates 17–61% and median duration of response of 7 months [50]. High dose intravenous methotrexate has resulted in overall response rates of 56% [55]. Recently, temozolamide is being extensively evaluated in phase I and II studies, either alone or in combination with other chemotherapeutic drugs (vinorelbine, cisplatin and capecitabine), for recurrent and progressive brain metastasis from solid tumors, including breast cancer [56, 57]. Theses studies have shown median survival time of 4–7 months.

8.4. Whole-Brain Radiotherapy Alone. WBRT is the main stay of treatment for most patients with brain metastasis, which produces symptomatic relief especially of headache and seizures in 75–80% of patients. It also improves survival to about 3–6 months and quality of life and radiological response in up to 60% of the cases [58]. For breast cancer patients, which responds better to WBRT and in patients with longer life expectancy (>6 months), a fraction size of less than 3 Gy is usually administered. Other side effects are alopecia, mild skin toxicity, fatigue, nausea, vomiting, and so forth. Late side effects are urinary incontinence and memory or cognitive disturbances. Late radiation-induced dementia is a rare occurrence, in only 1.9–5.1% of the patients [59].

8.5. Surgical Resection. Improved imaging and localization techniques have made surgery an accepted treatment option, particularly in patients with good prognostic factors. There is no direct evidence comparing WBRT alone versus surgery alone. Numerous retrospective studies have reported superiority of surgical resection over WBRT alone, but all of them had inherent selection bias, that is, patients selected for surgery had good performance status, single metastatic lesion, young age and so forth. The median survival in this good prognostic group is approximately 12 months, better than that for WBRT. Further, it has been estimated that only 30% of patients with brain metastases are suitable for surgery.

8.6. Stereotactic Radiotherapy. It involves the delivery of a single high-dose fraction of external radiation to a targeted lesion in the brain using multiple cobalt sources (gamma knife), modified linear accelerator (LINAC) or cyber knife. It has a potential to achieve high local control and is essentially used as a substitute for surgical treatment in patients with lesions less than about 3 cm in diameter. The good aspects of SRS are lack of discomfort, minimal invasiveness (no surgical incision), reduced hospitalization time (outpatient basis), with negligible damage to the surrounding healthy tissues. This stereotactic radiotherapy is ideal to target for sterotaxy, being small, spherical, well defined with distinct margins on contrast enhancement. These characteristics help to achieve conformal dose distributions with minimal damage to surrounding tissues. One of its greater advantages is that it can be targeted to those areas where surgical resection is not possible.

Whole-brain radiotherapy with radiosensitizers, intracavitary plus interstitial brain irradiations and chemoradiotherapy are under clinical trial these days and these approaches look promising for future management of brain tumor resulting from breast cancer metastasis.

9. Summary

Brain metastasis is a challenging clinical problem and a leading cause of death from cancer. Disruption of the blood-brain barrier was observed in triple-negative breast cancer and basal type breast cancer patients who developed breast cancer metastasis to the brain. Elucidation of the signaling pathways and processes that mediate the early steps of extravasation of breast tumor cells across brain microvascular endothelial cells should provide important information on the biology of tumor cell entry to the brain. Ultimately, this could lead to the design of better therapeutical approaches for blocking changes in permeability and integrity of the brain vasculature and inhibiting brain tumor angiogenesis and tumor growth.

Abbreviations

AJs: Adherens junctions
BBB: Blood-brain barrier
BMEC: Brain microvascular endothelial cells
BrMs: Brain metastasis signature
COX-2: Cyclooxygenase 2
CNS: Central nervous system
EC: Endothelial cells
ECM: Extra cellular matrix
EGFR: Endothelial growth factor receptor
FAs: Focal adhesion sites
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
HUVECs: Human umbilical vein endothelial cells
HBMECs: Human brain microvascular endothelial cells
JAM: Junctional adherens molecules
LCM: Laser capture microdissection
MMPs: Multiple matrix metalloproteinases
TJs: Tight junctions
TEER: Trans-endothelial electrical resistance
VEGF: Vascular endothelial growth factor
VEGFR: Vascular endothelial growth factor receptor.

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Review Article

Neurosurgical Treatment of Breast Cancer Metastases to the Neurocranium

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Breast cancer metastases to the neurocranium might involve the bone, the dura, or the brain parenchyma. The latter location is the far most common. The annual incidence of brain metastases in patients with breast cancer is in the range of 4–11 per 100.000 persons per year. Symptoms and findings mainly result from the location of the lesion. The diagnostic method of choice is magnetic resonance imaging before and after administration of contrast material. Breast cancer brain metastases present as solid, cystic, or partially cystic lesions with marked contrast enhancement and perilesional edema. The therapeutic option of choice is microsurgical resection whenever possible. Adjuvant treatment includes radiotherapy, radiosurgery, and/or chemotherapy.

1. Introduction

Breast cancer metastases to the neurocranium may involve the bone, the dura, or the brain parenchyma. The latter is the most common location. Affected patients are often in an advanced stage of disease [1]. According to advances in treatment of primary tumors, the amount of patients eligible for surgery is rising.

As early as in 1889, Stephen Paget showed that "the distribution of the secondary growths is not a matter of chance." Based on autopsy findings in 650 patients with breast cancer, he described affection of the cranium in 36 cases, whereas there was no single case of metastasis to the hand or feet [2]. It is an important clinical observation that tumors exhibit a predilection to metastasize to certain organs. Current research has addressed the molecular process of the metastatic cascade: (a) migration from the primary tumor, (b) dissemination into and survival in the blood vessels, (c) extravasation, and (d) proliferation at a distant site as well as the importance of the tumor-host interface [3–5].

In the first section of this paper, the general terms of neurosurgical treatment of metastases to the neurocranium from solid tumors are presented. In the second section, special issues of brain, bone, and dural metastases from breast cancer are discussed.

2. General Terms of Neurosurgical Treatment of Metastases to the Neurocranium

2.1. Clinical Examination. Diagnostic workup includes a complete neurological examination and evaluation of the extent of the primary tumor as well as comorbidity. Bone metastases might be detected by bulking of the calvaria. Brain metastases often lead to hemiparesis, ataxia, and aphasia [6]. Neurological examination can also give clues for possible spinal involvement. These patients might show ataxia and paresis and complain about radicular pain or sensation deficits.

2.2. Imaging. Magnetic resonance imaging (MRI) is useful for detecting small intraparenchymal lesions down to 1 mm diameter. Concerning this issue, MRI is highly superior to computed tomography (CT). Furthermore, MRI can sufficiently detect leptomeningeal spread and small dural lesions. It is very useful in detecting dural involvement in patients with bone or brain metastases. This information is important for surgery. MRI is usually generated in a sagittal, coronal, and axial view.

CT is useful in determining the extent of bone destruction, either in bone metastases or in bone involvement resulting from metastases to the dura or brain.

Scintigraphy is used for screening purposes. If metastasis to the skull is suspected, CT scan should follow. If metastasis to the brain is suspected, MRI should follow.

Figure 1 shows examples for imaging results in patients with breast cancer metastases to the neurocranium.

2.3. Neuronavigation. Nowadays, preparation for surgery includes neuronavigation for most cases of intracranial tumors. Neuronavigation is a 3D computer model of the patient's head which can be used intraoperatively as a reference location system. Basically, a thin-slice MRI or CT scan is performed after reference markers have been applied to the patient's head (*fiducial markers*). The 2D data set is transferred to a working station and processed into a 3D set. Immediately before starting the operation, the position of the patient's head is registered to the system in relation to a reference star. Thus, the surgeon can control the location of a pointer tip intraoperatively. Neuronavigation is helpful in minimizing the approach to the tumor. In glioma surgery, it is also useful for resection control. It must be noticed that intracranial structures are "shifting" after opening of the skull and again after resection of intracranial lesions. This incident is called *brain shift*.

3. Breast Cancer Metastases to the Neurocranium

3.1. Skull Metastases. The rate of hematogenous skull metastases, even though it is low in comparison to brain metastases, is higher in breast cancer than in many other tumors [7]. Hopkins et al. have described that bone metastases were detected by 99 mTc scintigraphy in approximately 50% of breast cancer patients in an early stage of the disease [8]. However, most of the lesions do not become symptomatic. It can be estimated that they grow slowly allowing other complications of the underlying breast cancer to develop. Most patients present with local swelling, sometimes accompanied by local pain. Neurological deficits are infrequent at the time of presentation. Bone metastases may reach significant size until they become symptomatic and, thus, until they are diagnosed. Metastases of the calvaria are often noted by the patient or by the patients family as swelling [9, 10]. Skull base metastases may become symptomatic by diplopia and/or exophthalmia when the orbit is involved. CT is required for visualization of the extent of bone destruction. MRI is superior to CT in detecting infiltration of the dura and neural tissue. Thus, both techniques are required preoperatively. Surgery should be considered when (1) a neurological deficit is present and/or (2) massive destruction of bone (and dura) occurs, (3) when there is a painful mass, (4) when solitary metastasis is present, or (5) when confirmation of the diagnosis is warranted [10]. Surgery aims to resect the infiltrated bone and replace it by bone-cement (cranioplasty). Alternatively, a titan mesh may replace the bony defect. This is especially useful in complex lesions involving the skull base. If the dura is infiltrated, it needs to be both resected and replaced The differential diagnosis of skull metastases includes primary skull tumors (e.g., osteoma, chondrosarcoma, chordoma, dermoid, and epidermoid cysts) and benign tumor-like lesions (e.g., fibrous dysplasia, hyperostosis, and eosinophilic granuloma) [10].

3.2. Dural Metastases. Dural metastases are uncommon. According to a postmortem series of 27 patients, breast cancer was the second most common malignancy to cause dural metastases. The most frequent cancer type was prostate cancer. The third most common type was cervical cancer [11]. Dural metastases may become symptomatic as solid masses or as metastatic subdural fluid collection, assembling a chronic subdural hematoma. Dural metastases are an important differential diagnosis of meningioma and must be suspected in every patient with chronic subdural hematoma and underlying malignant disease [12–14]. When solid dural metastases are removed, the dura is incised circumferentially around the lesion. Then, the tumor is dissected from the brain tissue with cottonoids and gentle coagulation. Retraction of the brain should be avoided. Accompanying veins should be preserved. After circumferential dissection, the tumor can be gently removed. The dura has to be replaced by autologous graft tissue or artificial material. The intraoperative aspect of dural metastases is quite similar to that of meningioma. Histological examination is essential to clarify the diagnosis.

3.3. Brain Metastases

3.3.1. Epidemiology. Breast cancer is the second most common solid tumor that forms brain metastases. The most common tumor type is lung cancer. The annual incidence of breast cancer brain metastases in USA is in the range of 5–10 per 100.000 per year. Altogether, 10–15% of patients with metastatic breast cancer will develop brain metastases during the course of the disease. The median age at time of diagnosis of breast cancer is 47 years. The mean latency between diagnosis of breast cancer and the detection of breast cancer brain metastases is 2-3 years. However, brain metastases may occur even as long as 20 years after diagnosis of breast cancer [1, 6, 15].

3.3.2. Location and Symptoms. Brain metastases characteristically grow at the white/gray matter border. They are more frequently located in the supratentorial than in the infratentorial space. Symptoms depend on the size and the exact location of the lesion. Even small lesions may cause neurological deficits if they grow inside or close to eloquent brain areas as the motor area or the speech regions. Large lesions may cause mass effect by increasing the intracranial pressure or by blocking cerebrospinal fluid (CSF) pathways causing hydrocephalus. According to our own data including 47 patients, the most frequent symptoms at the time of presentation to a neurosurgical unit were ataxia (23%), headache (21%), visual disturbance (15%), hemiparesis (11%), and

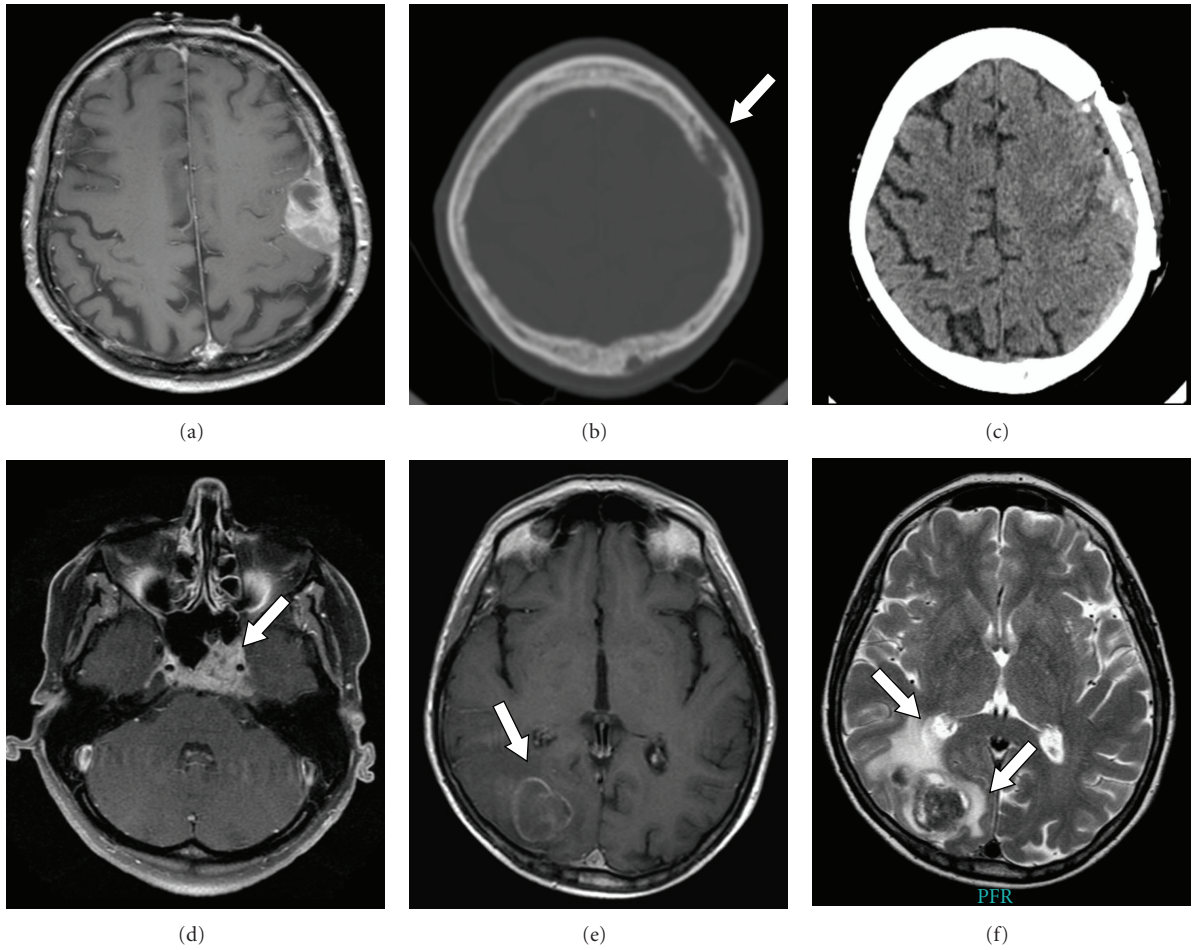

Figure 1: (a)–(c) 77-year-old femal with aphasia resulting from breast cancer dural metastasis, (a) preoperative T1-weighted MRI showing contrast enhancement of a left parietal dural tumor. (b) Preoperative CT scan shows bone erosion (arrow). (c) Postoperative CT scan documents the removal of the dural mass and the infiltrated bone which has been substituted by bone cement cranioplasty. (d) T1-weighted MRI showing contrast-enhancing breast cancer skull base metastasis in the clival region occurring in a 45-year-old female with known breast cancer and diplopia (arrow). (e, f) MRI of a 69-year-old female with right occipital breast cancer brain metastases. (e) T1-weighted MRI shows a ring-like contrast-enhancing lesion (arrow). (f) T2-weighted MRI shows the lesion with significant peritumoral finger-like edema (arrows).

vertigo (11%) [6, 16]. In few cases, brain metastases are incidental findings during staging procedures.

In 2005, we published a series of 177 consecutive patients who underwent craniotomy for newly diagnosed brain metastases from various tumors. Following lung cancer, breast cancer was the second most common origin of brain lesions. Colorectal cancer and renal cancer were the third and fourth most common origins. The amount of solitary brain lesions (only detectable distant metastases in the body) was 26% in breast cancer lesions whereas it was 72% in nonsmall cell lung cancer (NSCLC), 29% in colorectal cancer (CRC), and 56% in renal cancer (RC). Extracranial metastases were present in 54% of breast cancer patients versus 45% in NSCLC, 59% in CRC, and 33% in RC. Synchronous diagnosis of the primary tumor and brain metastasis is very uncommon in breast cancer (3%, 1 patient). In contrast, it is common in NSCLC (54%) and CRC (59%). It is uncommon in renal cancer (11%) [6].

3.3.3. Prognostic Indicators. Overall, accepted indicators of prolonged survival are only younger age and good patient performance. There is no uniform definition of elderly patients with brain metastases. Usually, the threshold between younger and elderly individuals is set somewhere between 50 and 75 years [17–19]. In a systematic statistical evaluation, we could define the age threshold relevant for prognostic differences as 65 years [16]. Patient performance is usually documented as Karnofsky performance score (KPS) as described by Karnofsky and Burchenal, in 1949 [18, 19]. Usually, a KPS of 70 or higher is regarded as favorable and is often required for the inclusion of patients into treatment studies.

For clinical decision making, additional factors are taken into account. Herein, local control of the primary tumor is an important factor as well as the extent of extracranial metastases and the number of intracranial metastases [20–22]. Figure 2 shows the survival curve of 47 patients who

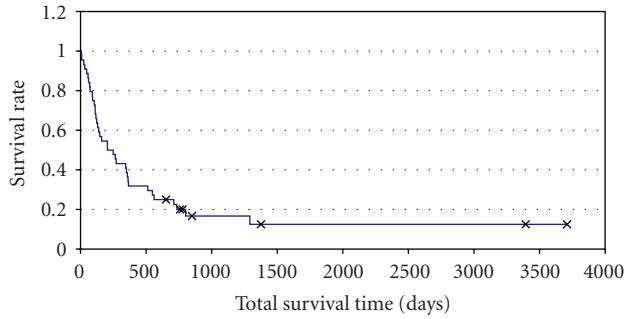

Figure 2: Kaplan Meier plots depict survival of 47 patients with breast cancer brain metastases who underwent surgical resection and adjuvant radiotherapy in our department between 1994 and 2004. 42 patients had one brain metastases, 4 patients had 2 intraparenchymal lesions, and 1 patient had 3 metastases. Median survival was 205 days = 29 weeks.

underwent surgery for breast cancer brain metastases in our department in a 10-year interval.

3.3.4. Imaging. The standard imaging technique for the detection of brain metastases is MRI before and after the administration of contrast material. Herein, brain metastases appear as ring-like, solid or partially solid/cystic contrast-enhancing lesions in T1-weighted imaging. The surrounding edema, which is often significant, can be best visualized in the T2-weighted image. It is a finger-like edema of the white matter. MRI is highly superior to (CT) in detecting small lesions down to 1 mm diameter. CT is adequate for the emergency situation and should be followed by MRI once brain metastases are suspected. Infrequently, patients with brain metastases present with acute, stroke-like symptoms caused by intratumoral bleeding. This condition is relatively frequent in renal cancer and malignant melanoma but may also occur in individuals with breast cancer.

3.3.5. Surgery: Indications and Techniques. The goal of surgery in patients with brain metastases is to establish the diagnosis and relieve mass effect. If brain metastases are accessible, they should be completely removed. Currently, there is evidence that brain metastases, like gliomas, create an infiltration zone involving the adjacent brain parenchyma. It will be up to further studies to generate treatment decisions from this observation.

Randomized prospective data concerning the surgical treatment of patients suffering from brain metastases are only available for patients with single brain lesions. Herein, it has been proven that surgery plus radiotherapy (external beam radiation) is superior to radiotherapy alone in prolonging patient's survival [23, 24].

Sufficient data for patients with multiple metastases are lacking. From the existing literature, it can be assumed that in patients with 1–3 brain lesions open microsurgical resection should be considered whenever possible. In this situation, surgical intervention is indicated in patients who present with (1) a primary tumor under control, (2) accessible brain lesions, and (3) a total number of brain metastases of at least 3 [18, 19]. As a rule of thumb, patients undergoing surgical resection of brain metastases should have a life expectancy of at least 6 months. However, occasionally, patients with multiple brain metastases present with one life-threatening lesion. This lesion might be resected in an emergency operation in order to prevent sudden patient death. In general, due to advances in operative techniques and neuroanesthesia, the removal of 1–3 brain metastases in 1-2 operations is appropriate. Small lesions ($\leq$3 cm diameter, $\leq$3 metastases in total) might be treated by stereotactic radiosurgery as an alternative to open surgery or as additional treatment [6, 18, 19, 25].

In patients with multiple metastases, it has been shown that radiotherapy plus radiosurgery is superior to radiosurgery alone. As a consequence, radiosurgery should not be performed as single therapeutic method [26].

Preparation for surgery includes the selection of the approach which is in most times neuronavigation. Corticosteroids should be given to prevent or reduce brain swelling. The patient's head is placed in a rigid fixation system to prevent unwanted movement during the operation. Neuronavigation-guided skin incision and bone trepanation is performed. In superficial metastases, the dura should be inspected for possible infiltration. Infiltrated dura needs to be replaced. The intracranial part of the operation is carried out using the operative microscope. After dural opening, the metastasis is accessed and dissected from the surrounding brain tissue. In larger lesions, it may be necessary to partly remove the lesion before dissecting it. Frozen sections may be performed for intraoperative histology. Completely removing the metastasis without damaging the adjacent brain tissue should always be the goal. After removal of the lesion, meticulous hemostasis is performed by cauterization followed by the application of cellulose strips. After irrigation with saline, the dura is closed and the bone flap is placed and fixed. Tissue specimens are sent to histopathology for definitive diagnosis.

3.3.6. Complications of Surgery. Brain metastases tend to cause brain swelling which can be treated by steroids and osmotic diuresis. Steroids should be continued over a few days after surgery and should then be reduced over some days before they are tapered. Prophylactic antibiotics can reduce infection. Careful microsurgical dissection prevents injury to the brain tissue and to small veins thus preventing unfavorable neurological outcome. Specific complications of surgery include intracranial bleeding (intraparenchymal, epidural, subdural), subtotal resection (if it is not primarily wanted), injury to eloquent brain areas and cerebrospinal fluid fistula. In patients who have had seizures prior to surgery, anticonvulsants should be given. Postoperatively, special care should be taken to prevent deep vein thrombosis.

3.3.7. Complications of Brain Metastases: The Course of the Disease. As many as 50% of patients will suffer from brain metastasis recurrence—either at the site of the treated metastasis or anywhere else within the brain. Reoperation is

possible. However, most patients fail to qualify for repeated surgery due to poor performance.

Patients with breast cancer brain metastases tend to develop leptomeningeal dissemination (leptomeningeal carcinomatosis) more often than patients with brain metastases of other origin. Leptomeningeal spreading is caused by dissemination of tumor cells via the cerebrospinal fluid (CSF). It is best visualized with contrast-enhanced MRI. Treatment might consist in intrathecal chemotherapy given via an implanted reservoir. Herein, methotrexate is commonly used [27]. Radiotherapy might be an alternative or additional palliative option [28].

3.3.8. Adjuvant Treatment. Radiotherapy is applied on a routine basis after surgical resection. Radiosurgery is an option for small lesions ($\leq$3 cm diameter) either as an alternative to open resection or as additional treatment.

Chemotherapy for brain metastases is mostly targeted at the primary tumor. Delivering systemic chemotherapy to brain metastases is highly limited by the presence of the blood-brain barrier. Recent evidence highlights the importance of the alkylating agent temozolomide in patients with newly diagnosed brain metastases from breast cancer [29]. Temozolomide is applied orally and is well tolerated even by elderly patients. It is widely applied in the treatment of malignant gliomas.

3.3.9. Postoperative Followup. Patients with brain metastases should be followed closely by a neuro-oncology/neurosurgery unit. Following surgery and radiotherapy, we look after patients in our outpatient department every three months. Herein, clinical examination and cranial MRI are performed. If brain metastasis recurrence is noted, recraniotomy is discussed. Further options include radiosurgery and chemotherapy depending on the primary tumor.

4. Conclusions

Breast cancer metastases to the neurocranium might involve the bone (either the calvaria or the skull base), the dura (either as a solid mass or as subdural fluid collection), or the brain parenchyma. The latter location is the far most common. The diagnostic method of choice is magnetic resonance imaging. The treatment consists in neuronavigation-guided microsurgical removal whenever possible. The decision whether to operate depends mainly on the stage of the disease, the number of brain lesions, and the performance status of the patient. Postoperative treatment consists in radiotherapy and/or chemotherapy depending on the primary tumor. Radiosurgery may be an alternative to surgery or can be added to surgical treatment in selected cases.

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