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ARTICLE

Breast cancer global tumor biomarkers: a quality assurance study of intratumoral heterogeneity

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Abstract

Biomarker analysis of invasive breast carcinoma is useful for prognosis, as surrogate for molecular subtypes of breast cancer, and prediction of response to adjuvant and neoadjuvant systemic therapies. Breast cancer intratumoral heterogeneity is incompletely studied. Comprehensive biomarker analysis of estrogen receptor (ER), progesterone receptor (PR), HER2, and Ki67 labeling index was performed on each tissue block of 100 entirely submitted breast tumors in 99 patients. Invasive carcinoma and in situ carcinoma was scored using semiquantitative histologic score (*H*-score) for ER and PR, HER2 expression from 0 to 3+, and percentage positive cells for Ki67. Core biopsy results were compared with surgical excision results, invasive carcinoma was compared with in situ carcinoma, and interblock tumoral heterogeneity was assessed using measures of dispersion (coefficient of variation and quartile coefficient of dispersion). Overall concordance between core biopsy and surgical excision was 99% for ER and 95% for PR. Mean histologic score of ER was significantly lower in invasive carcinoma between core biopsy and surgical excision ($p = 0.000796$). Intratumoral heterogeneity was higher for PR than for ER (mean coefficient of variation for ER 0.08 stdv 0.13 vs. PR 0.26 stdv 0.41). Ki67 labeling index was significantly higher in invasive carcinoma as compared with associated ductal carcinoma in situ on surgical resection specimen ($p \le 0.0001$). Ki67 hotspots were identified in 47% of cases. Of 52 HER2 negative cases on core biopsy, 10 were scored as equivocal on surgical resection. None (0/10) were amplified by *Her-2/neu* fluorescence in situ hybridization. Overall, biomarkers on core biopsy showed concordance with the surgical excision specimen in the vast majority of cases. Biomarker expression of in situ closely approximates associated invasive carcinoma. Intratumoral heterogeneity of PR is greater than ER. Biomarker expression on diagnostic core biopsy or single tumor block is representative of breast carcinoma as a whole in most cases and is appropriate for clinical decision-making.

Introduction

Biomarker analysis of invasive breast carcinoma is required for understanding the biology of a patient's disease. Routinely performed tests include immunohistochemistry for estrogen receptor (ER), progesterone receptor (PR), and Ki67, a proliferation marker, along with either immunohistochemistry and/or fluorescence in situ hybridization (FISH) for HER2 protein overexpression/gene amplification. These biomarkers are commonly used both to guide treatment using targeted therapies, such as hormonal treatments and anti-HER2 agents, and as surrogates for the molecular classification of breast tumors, which has prognostic significance [1–3].

Biomarker testing is most commonly performed on the diagnostic core biopsy specimen, which has advantages including rapid tissue fixation and the ability to use the results for systemic therapy planning, including administration of neoadjuvant systemic therapy, which has been shown to be equivalent to adjuvant administration, may allow for less extensive surgery and also enables in vivo observation of response to treatment [4, 5]. Concordance between core biopsy specimens and subsequent resection has been shown in multiple studies [6–16]. Testing on the resection specimen may be performed if invasive carcinoma is found in a surgical specimen performed for other reasons, if HER2 testing was equivocal on the core biopsy, or at the pathologist's discretion if multiple foci, variable

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morphology, or other unusual situations suggest that repeat testing may be of value. In other words, the pathologist may consider the impact of morphologic heterogeneity.

Intratumoral heterogeneity is of great interest because the presence of different clones within a tumor may impact our ability to provide personalized cancer treatment. Current recommendations include reporting of ER and PR as positive if ≥1% of the tumor cells show reactivity by immunohistochemistry, and reporting of HER2 as positive if >10% of tumor cells show strong, complete membranous reactivity [17, 18]. If targeted treatments are employed, the presence of heterogeneity could impact response.

We were interested in assessing heterogeneity of the common biomarkers through comprehensive histologic examination and biomarker analysis of a group of consecutive invasive breast carcinomas with gross tumor size of 3 cm or less. Invasive carcinoma was entirely submitted for histologic examination and ER, PR, HER2, and Ki67 immunohistochemistry was performed on each tumor block showing invasive carcinoma. This comprehensive analysis, which to our knowledge is the first of its kind, allowed us to study concordance of core biopsy results with entire tumor on surgical resection, concordance between biomarkers in invasive and in situ carcinoma, and spatial heterogeneity among tumor blocks of entire tumors.

Methods

Following approval by the Institutional Review Board at the University of Pittsburgh, 101 consecutive breast surgical resection specimens with prior percutaneous core biopsy diagnosis of invasive breast carcinoma were selected. Grossly identifiable tumor was entirely submitted for histologic examination. American Society for Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines were followed for tissue processing, including cold ischemic time and formalin-fixation time [18]. On each tumor block, routine hematoxylin and eosin stained slides were prepared. Following completion of the routine surgical pathology examination, immunohistochemical stains were performed on each block demonstrating invasive carcinoma. Immunohistochemical stains consisted of estrogen receptor (ER)(Clone SP1, Ventana), progesterone receptor (PR) (Clone 1E2, Ventana), Her-2/neu (HER2) (Clone 4B5, Ventana), and Ki67 (Clone 30-9, Ventana).

Immunohistochemistry scoring for each biomarker was performed on the in situ and invasive carcinoma components on each tumor block and on the available core biopsy specimens. ER and PR were scored by modified histologic score (*H*-score) method with score ranging from zero (completely negative) to 300 (100% of tumor cells strongly positive). The histologic score is determined by multiplying the intensity of expression (0–3) by the percentage of cells showing that intensity (0–100%). The sum of these numbers is referred to as the histologic score. For this study, a histologic score ≥ 1 was considered positive. Histologic score 201–300 was considered as "strong" expression, 101–200 was considered as "moderate" expression, and 1–100 was considered as "weak" expression.

HER2 immunohistochemistry was scored per the FDA-cleared Interpretation Guide for Ventana anti-Her-2/neu antibody clone 4B5, with scores of 0 and 1 considered together as negative, score of 2 as equivocal, and score of 3 as positive. Ki67 labeling index was scored as a percentage of cells with staining of any intensity, and recorded as both an overall percentage of positive cells on each slide as well as a percentage of positive cells in "hotspots", if applicable. Because hotspots are considered as areas with "particularly prevalent" Ki67 staining, and there is no consensus quantitative definition of hotspot, we chose to define hotspots as areas in which Ki67 staining was at least 10% higher than the average over the whole slide. At our institution, a specific "cut-off" value is not used for consideration of more aggressive treatment. Rather, Ki67 is used as a continuous variable in determination of the Magee Equations™. For the purposes of this study, Ki67 labeling index in individual blocks was compared to the labeling recorded on core biopsy specimen and cut-off of 25% was used [19]. Age, maximum tumor size, tumor type, and tumor grade were also recorded.

Statistical analysis was performed in STATISTICA (StatSoft, Inc., Tulsa, OK, USA). The *p*-values were calculated using Wilcoxon-matched pairs test. The results were considered statistically significant at the level of 1% ($p < 0.01$). Heterogeneity of reactivity among tissue blocks for ER, PR, and Ki67 was analyzed by calculating coefficient of variation and quartile coefficient of dispersion. Kruskal–Wallis test analysis of variance (ANOVA) was performed to compare ER, PR, and Ki67 expression by tumor stage, by combining T1a and T1b into one group, and comparing with T2 and T3 tumors in the study cohort.

To analyze HER2, contingency tables with frequency distribution of the paired groups were constructed. Negative scores (0 and 1+) were combined in the analysis. To compare resection specimens showing heterogeneity of HER2 immunohistochemical expression with core biopsy results, the most clinically significant result was assigned. For example, if five tumor blocks were submitted, and the HER2 immunohistochemical results were 0, 1+, 2+, 2+, and 3+, on individual tumor blocks, a positive result (3+) would be assigned for the case.

Results

The analysis included 100 invasive breast carcinomas from 99 patients ranging from 33 to 92 years old (mean 59 years).

Patient Age Mean: 60 years Median: 60 years Range: 33-92 years
Surgical Resection Procedure Segmental mastectomy: 70 (70%) Nipple-sparing mastectomy: 2 (2%) Modified radical mastectomy: 5 (5%) Skin-sparing mastectomy: 8 (8%) Total mastectomy: 15 (15%)
Nottingham Tumor Grade 1: 13 (13%) 2: 54 (54%) 3: 33 (33%)
Histology Invasive ductal carcinoma: 89 (89%) Invasive lobular carcinoma: 6 (6%) Mucinous carcinoma: 4 (4%) Metaplastic carcinoma: 1 (1%)
Tumor Phenotype by Immunohistochemistry and Her-2/neu FISH (Based on 96 core biopsy results available) ER+/PR+/HER2 Negative: 76 (79%) ER+/PR-/HER2 Negative: 4 (4%) ER+/PR-/HER2 Positive: 2 (3%) ER-/PR-/HER2 Negative: 9 (9%) ER+/PR+/HER2 Positive: 4 (4%) ER+/PR+/HER2 Equivocal: 1 (1%)
Ki67 Labeling Index (Based on 82 core biopsy results available) Low (0-10%): 19 (23%) Moderate (11-25%): 28 (34%) High (26-50%): 17 (21%) Very High (51-100%): 18 (22%)

Fig. 1 Clinical and tumor characteristics

Clinical and tumor characteristics and surgical management are reported in Fig. 1. Two cases had been excluded because the tumors were not entirely submitted for histologic examination as per the study protocol. In one patient, invasive breast carcinoma was identified in both the right and left breasts, and these tumors were analyzed separately. In three cases, multiple foci of invasive carcinoma with similar histopathologic morphology and same Nottingham tumor grade were identified in the same region of the breast. These foci were entirely submitted and analyzed together. Mean tumor size was 1.9 cm (SD 0.849), with tumor size ranging from 0.6 to 6.0 cm, with 8 tumors classified as T1a/T1b, 47 classified as T1c, 44 classified as T2, and 1 tumor classified as T3, and tumor blocks required to entirely submit invasive carcinoma in each case ranged from 1 to 22 with median 4.6 tumor blocks per case (Fig. 2).

Results of ER, PR, and Ki67 expression patterns in core biopsies and surgical resection specimens for invasive and in situ carcinoma are reported in Table 1. Using ANOVA (Kruskal–Wallis test), no statistically significant differences were observed in ER, PR, or Ki67 when comparing tumors by pathologic T stage (Table 2).

Correlation between core biopsy and surgical resection specimen

In invasive carcinoma, ER histologic score was available on the core biopsy specimen for 98 cases and on the surgical resection specimen in 100 cases. Mean ER histologic score was 232 (0–300, SD 89) on the core biopsy and 220 (0–300, SD 88) on the surgical resection specimens. In eight cases, invasive carcinoma was completely negative (ER histologic score 0) on both core biopsy and surgical resection. Using Wilcoxon-matched pairs test, a statistically significant difference was observed between mean ER histologic score on core biopsy and resection in invasive carcinoma ($p = 0.000796$). Concordance between core biopsy and surgical resection specimen for invasive carcinoma was 99% for ER positivity overall, however, one case (1%) with histologic score 0 on core biopsy had mean histologic score of 4 on the surgical resection specimen.

In invasive carcinoma, PR histologic score was available on the core biopsy specimen for 98 cases and on the surgical resection specimen in 100 cases. Mean PR histologic score was 149 (0–300, SD 103) on the core biopsy and 134 (0–291, SD 97) on the surgical resection specimens. In 18 cases, invasive carcinoma was completely negative (PR histologic score 0) on both core biopsy and resection specimen. No statistically significant difference between PR histologic score in invasive carcinoma on core biopsy and resection was observed ($p = 0.02253$). Concordance between core biopsy and surgical resection specimen was 95% for PR positivity in invasive carcinoma overall. Two cases with weakly positive PR histologic scores on core biopsy were negative on the surgical resection specimen, while 3 cases were negative (histologic score 0) on the core biopsy but carcinoma was weakly positive on surgical specimen (histologic scores 2, 6, and 66).

HER2 immunohistochemical scores were available in core biopsy/resection pairs for invasive carcinoma in 86 cases. In 12 cases, only HER2 FISH results were available, and in 2 cases, HER2 result was not available on the core biopsy specimen. These cases were excluded from comparison with the resection specimen.

On the core biopsy specimens, 52 cases were negative (immunohistochemistry score of 0 or 1+), 30 cases were equivocal (immunohistochemistry score 2 +), and 4 were positive (immunohistochemistry score 3+). Concordance rates with HER2 immunohistochemistry for invasive

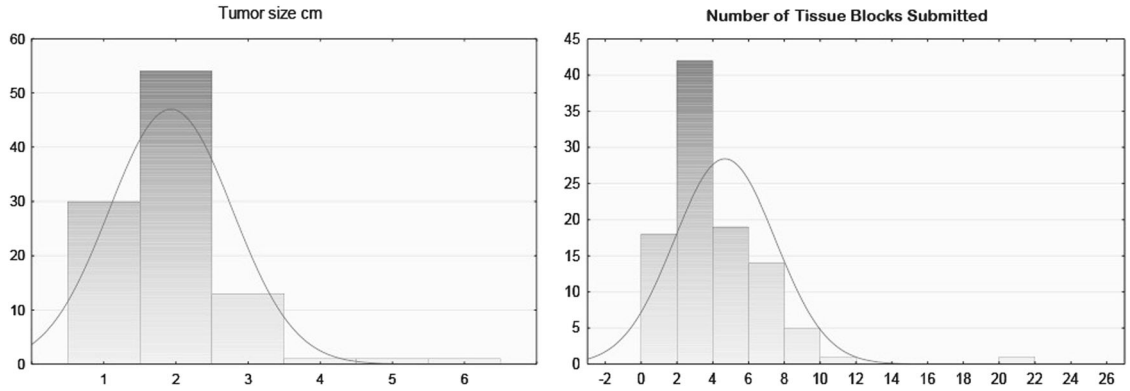

Fig. 2 Distribution of cases by final tumor size and number of tissue blocks required to entirely submit invasive carcinoma

carcinoma on resection specimen are reported in Table 3. Concordance was 100% for cases reported as positive on core biopsy. Of the 30 equivocal (HER2 2+) cases on core biopsy, one case was found to have HER2-positive areas on the resection specimen. *Her-2/neu* FISH had been reported as positive for amplification on the core biopsy in this case. Four cases were scored as HER2 0 or 1+ on the resection specimen. Of the cases that were HER2 negative on the core biopsy, no cases were positive (3+) by immunohistochemistry on the resection specimen. In 10 cases, HER2 was negative (0 or 1+) by immunohistochemistry in invasive carcinoma on the core biopsy specimen, but showed equivocal (2+) expression on at least one tumor block of the surgical resection specimen. *Her-2/neu* FISH was performed on a tissue block showing the strongest equivocal (2+) expression in these cases. Using the 2013 ASCO/CAP criteria, *Her-2/neu* FISH demonstrated that 9/10 cases were negative for amplification by both *HER-2/neu*/cep17 ratio and absolute *Her-2/neu* gene copy number, while 1 case was negative for amplification by *HER-2/neu*/cep17 ratio (ratio 1.16) but equivocal by *Her-2/neu* gene copy number (4.3 copies per cell) [17].

In invasive carcinoma, Ki67 immunohistochemical stain was available on the core biopsy specimen for 82 cases and on the surgical resection specimen in 100 cases. Mean Ki67 was 31% (1–95, stdv 26) on the core biopsy and 26% (1–96, stdv 26) on the surgical resection specimens. No statistically significant difference was observed between Ki67 proliferation using Wilcoxon-matched pairs test when comparing Ki67 proliferation in invasive carcinoma on core biopsy and resection ($p = 0.010931$). Using a cut-off value of 25% to compare individual blocks to the core biopsy results, differences were observed in 20/82 cases. In 12 cases, core biopsy showed Ki67 labeling index > 25% and at least one tumor block showed Ki67 labeling index ≤ 25%. In 8 cases, core biopsy Ki67 labeling index was ≤ 25%, but at least one tumor blocks showed Ki67 labeling index > 25%. These data are shown in Table 4.

Correlation of in situ and invasive carcinoma on surgical resection

On the surgical resection, ductal carcinoma in situ was identified in association with invasive carcinoma on the ER immunohistochemical stain in 79 cases, with mean ER histologic score 228 (0–300, stdv: 85). In five cases, ductal carcinoma in situ was completely negative (ER histologic score 0) on both core biopsy and resection specimen. Overall concordance between ductal carcinoma in situ and invasive carcinoma was 99%, with one case showing negative ductal carcinoma in situ and weakly positive invasive carcinoma (mean histologic score 94). There was no statistically significant difference between ER histologic score using Wilcoxon-matched pairs test when comparing ductal carcinoma in situ and invasive carcinoma ($p = 0.642504$).

Ductal carcinoma in situ was identified in association with invasive carcinoma on the PR immunohistochemical stain on 78 of the surgical resection specimens, with mean PR histologic score 138 (0–295, SD 96). In 15 cases, ductal carcinoma in situ was completely negative (PR histologic score 0) on both core biopsy and resection specimen. Overall concordance between ductal carcinoma in situ and invasive carcinoma was 88%. No statistically significant difference was observed using Wilcoxon-matched pairs test between PR histologic score when comparing expression of ductal carcinoma in situ and invasive carcinoma ($p = 0.825893$). In three cases, ductal carcinoma in situ was weakly positive and associated invasive carcinoma was negative.

Ductal carcinoma in situ was identified on the HER2 slides in 74 of the surgical resection specimens.

Table 1 Estrogen receptor, progesterone receptor, and Ki67 expression in core biopsy and surgical resections in invasive and in situ carcinoma

	Number of cases	Mean	Minimum	Maximum	Standard deviation
Estrogen receptor *H*-score					
Core in situ	17	263	205	300	28
Resection in situ	79	228	0	300	85
Core invasive	98	232	0	300	89
Resection invasive	100	220	0	300	88
Progesterone receptor *H*-score					
Core in situ	19	137	0	280	89
Resection in situ	78	138	0	295	95
Core invasive	98	149	0	300	103
Resection invasive	100	135	0	292	97
Ki67 labeling index					
Core in situ	14	22	1	75	23
Resection in situ	76	19	0.5	95	24
Core invasive	82	32	1	95	26
Resection invasive	100	27	1	97	26

Table 2 Mean estrogen receptor *H*-score, progesterone receptor *H*-score, and Ki67 labeling index by tumor stage

Stage	*N*	ER mean *H*-score (stdv)	PR mean *H*-score (stdv)	Ki67 mean labeling index (stdv)
T1a or T1b	8	224 (107)	132 (120)	26 (31)
T1c	47	211 (97)	137 (97)	28 (26)
T2	44	228 (76)	135 (96)	27 (24)
T3	1	284	20	7
Total	100	220	134	27
p-Value		0.6056	0.8429	0.7448

Overall, ductal carcinoma in situ was positive (HER2 score 3+) in 7 cases, equivocal (HER2 score 2+) in 38 cases, and negative (HER2 score 0 or 1+) in 28 cases, while invasive carcinoma was positive in 7 cases, equivocal in 33 cases, and negative in 34 of cases. Combining positive and equivocal cases, and using McNemar's χ^2 test, no statistically significant difference was observed (two-tail test, $p = 0.0614$), although 11 cases that were positive or equivocal in ductal carcinoma in situ for HER2 were scored as negative in the associated invasive carcinoma (Table 5).

Ductal carcinoma in situ was identified in association with invasive carcinoma on the Ki67 immunohistochemical stain for 76 of the surgical resection specimens, with mean Ki67 of 19% (0.5–95, stdv 24). A statistically significant difference was observed between ductal carcinoma in situ and invasive carcinoma on surgical resection specimen ($p \leq 0.0001$), with significantly lower proliferation in ductal carcinoma in situ.

Table 3 HER2 immunohistochemistry concordance between core biopsy and surgical resection specimens for invasive carcinoma

	Surgical resection			
Core biopsy	Positive	Negative	Equivocal	
HER2 result				
Positive	4	0	0	4
% Column	80.0%	0.0%	0.0%	
% Row	100.0%	0.0%	0.0%	
% Total	4.6%	0.0%	0.0%	4.6%
Negative	0	42	10	52
% Column	0.0%	91.3%	28.6%	
% Row	0.0%	80.8%	19.2%	
% Total	0.0%	48.8%	11.6%	60.5%
Equivocal	1	4	25	30
% Column	20.0%	8.7%	71.4%	
% Row	3.3%	13.3%	83.3%	
% Total	1.2%	4.7%	29.0%	34.9%
Total	5	46	35	86
% Total	5.8%	53.5%	40.7%	

Intratumoral heterogeneity of estrogen receptor, progesterone receptor, HER2, and Ki67 in invasive carcinoma in entirely submitted surgical resection specimens

Mean coefficient of variation for ER expression in individual cases among tissue blocks in the surgical resection specimens was 0.08 with stdv 0.13. Coefficient of variation ranged from 0 to 0.74 in individual cases. Mean quartile coefficient of dispersion was 0.04 with stdv 0.07. Quartile coefficient of dispersion ranged from 0.0 to 0.4 in individual

Table 4 Cases with discordant Ki67 between core biopsy and individual tumor blocks (using 25% cut-off) with corresponding estrogen receptor, progesterone receptor, and HER2 immunohistochemical score results

	Core biopsy	Individual tumor blocks			
Case number	Ki67 LI	Ki67 labeling index	ER *H*-score	PR *H*-score	HER2 score
1	30				
		20	200	0	1
		20	260	0	1
		15	210	0	1
		15	210	0	1
3	35				
		5	300	27	0
		5	300	0	0
		10	280	18	1
		10	270	0	1
		5	270	55	1
4	35				
		10	280	220	2
		15	240	220	2
		20	240	195	2
		20	240	180	2
		20	240	210	2
		20	240	210	2
9B	30				
		25	200	250	2
		12	220	200	2
14	35				
		20	240	215	1
		20	245	215	2
15	30				
		25	195	115	1
		45	165	63	2
		20	180	50	2
		35	100	0	2
23	20				
		30	250	0	2
		30	260	0	2
		20	230	55	2
24	30				
		45	295	0	1
		30	290	0	1
		35	290	0	1
		35	295	0	1
		35	290	0	1
		35	290	0	1
		45	295	0	1
		35	285	0	1
		45	290	0	1
24		40	290	0	1
		35	290	0	1
		40	290	0	1
		30	290	0	1
		40	295	0	1
		25	290	0	1
		35	290	0	1
		30	285	0	1
		35	290	0	1
		35	290	0	1
		40	295	0	0
28	10				
		25	215	220	1
		25	210	210	2
		30	250	210	2
		30	230	210	2
		25	210	200	2
		25	180	195	2
		30	190	180	2
		25	195	180	2
36	15				
		30	215	220	0
		30	170	200	0
		25	190	230	0
		30	160	220	0
		35	180	215	0
		40	180	250	0
		40	185	210	0
42	20				
		30	260	150	1
		25	180	110	2
		30	210	150	1
56	60				
		20	270	0	2
		30	290	0	2
		30	270	0	2
		40	270	0	2
		40	270	0	2
		40	290	0	2
		30	290	0	2
		30	295	0	2
		30	295	0	2

Table 4 (continued)

Case number	Core biopsy Ki67 LI	Individual tumor blocks Ki67 labeling index	ER *H*-score	PR *H*-score	HER2 score
		30	295	0	2
58	35				
58		25	280	160	0
		30	280	140	1
		30	271	160	2
		30	271	140	1
		30	265	140	1
		25	271	140	0
		35	280	120	1
61	25				
		50	280	160	0
		60	280	150	0
62	10				
		15	290	230	1
		15	280	230	0
		30	260	240	0
67	40				
		40	290	165	1
		35	270	145	2
		15	285	210	1
		15	265	215	1
		12	290	210	1
72	28				
		20	280	240	2
		15	280	210	2
		25	280	190	2
86	35				
		20	290	190	1
		30	290	105	2
		20	205	0	2
		30	280	70	2
		30	280	23	2
		15	280	53	2
		30	290	90	2
		20	295	0	1
92	10				
		5	261	250	2
		30	275	240	2
		10	245	240	2
		5	230	210	1
		5	273	160	2
		8	273	240	2
93	20				
		30	290	40	1
		15	290	21	1

Table 5 HER2 concordance between in situ and invasive carcinoma by immunohistochemistry on surgical resection specimens

	Invasive carcinoma		Total
	HER2-positive or equivocal	HER2 negative	
In situ carcinoma			
Positive or equivocal	35	11	46
HER2 negative	3	25	28
Total	38	36	74

cases (Fig. 3). No cases that were identified as ER-positive overall on the resection specimen showed individual tumor blocks that were completely ER-negative in the resection specimens.

Mean coefficient of variation for PR expression among tissue blocks in the surgical resections specimens was 0.26 with stdv 0.41. Coefficient of variation ranged from 0.0 to 2.25 in individual cases. Mean quartile coefficient of dispersion was 0.14 with stdv 0.24. Quartile coefficient of dispersion ranged from 0.0 to 1.0 in individual cases. (Fig. 4) In contrast to ER results, in ten cases with positive PR overall, completely PR negative tumor blocks were identified, with 4 cases showing one PR negative tumor block and 5 cases showing two PR negative tumor blocks, and one case showing 4 negative tumor blocks. The details are reported in Table 6, and appear to be largely isolated to PR when also comparing the ER, Ki67, and HER2 results for these blocks to the remainder of the tumor blocks. This likely reflects the greater heterogeneity of PR expression observed as compared with ER. The quartile coefficient of dispersion for PR was 3.5 times as great (0.14/0.04) as that for ER.

Overall designation for HER2 in invasive carcinoma on resection specimen was positive in 8 cases, equivocal in 41 cases, and negative in 51 of cases.

In observing individual tumor blocks, HER2 immunohistochemical expression showed complete agreement among tumor blocks in 77 cases, showed both negative (0 or 1+) and equivocal (2+) blocks in 20 cases, and showed both equivocal (2+) and positive (3+) blocks in 2 cases. One case showed a spectrum of HER2 expression patterns including negative (1+) on one block, equivocal (2+) on

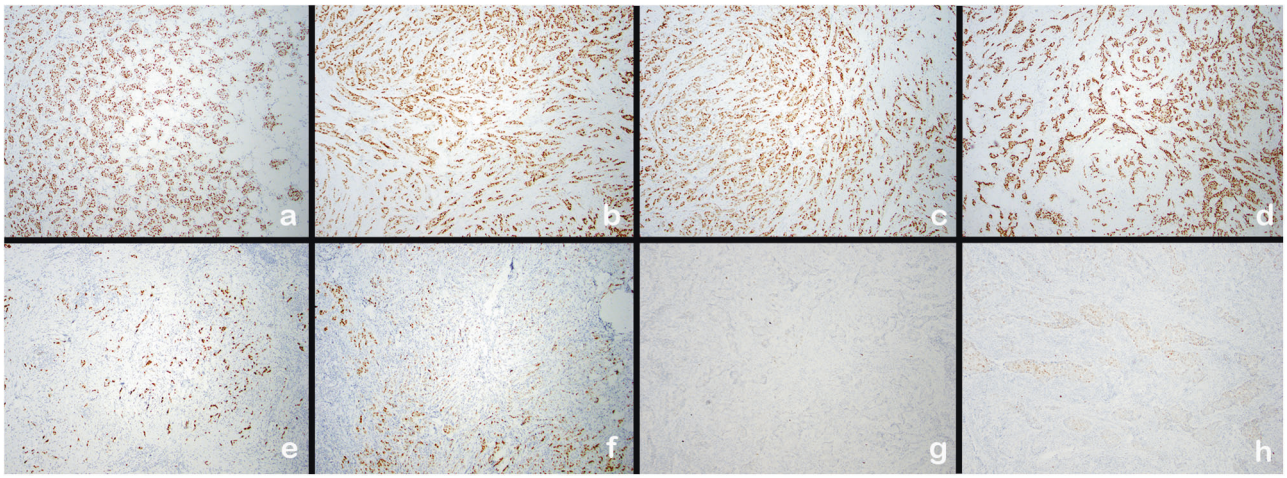

Fig. 3 Intratumoral variation of estrogen receptor (ER) immunohistochemical expression: **a–d** Case 29 (low variation)—individual tissue blocks with histologic scores of 290, 280, 280, and 280, respectively (coefficient of variation 0.02, quartile coefficient of dispersion 0.00), **e–h** Case 39 (high variation)—individual tumor blocks with histologic scores of 210, 105, 45, and 70, respectively (coefficient of variation 0.74, quartile coefficient of dispersion 0.40)

two blocks, and positive (3+) on two blocks (Fig. 5). The core biopsy result was not available in this case.

Ki67 hotspots were identified in invasive carcinoma in 47 (47%) of cases. Using 10% as the minimum difference between areas of increased proliferation on individual tumor blocks, the difference between mean Ki67 proliferation for hotspots and mean Ki67 for whole slide average ranged from 5 to 35%, with mean difference of 16%. Mean coefficient of variation for Ki67 labeling index among tissue blocks in the surgical resections specimens was 0.24 with stdv 0.22. Coefficient of variation ranged from 0.0 to 0.97 in individual cases. Mean quartile coefficient of dispersion was 0.12 with stdv 0.12. Quartile coefficient of dispersion ranged from 0.0 to 0.62 in individual cases.

Discussion

In this study of 100 invasive breast carcinomas from 99 patients, we performed a comprehensive analysis of the key biomarkers used in breast cancer systemic treatment decisions in tumors ranging from 0.6 to 6.0 cm. We focused on concordance of the global biomarker status with core biopsy findings, comparison of invasive carcinoma with associated in situ carcinoma, and analysis of heterogeneity of biomarker expression among tumor blocks.

The preoperative percutaneous core biopsy is the preferred method for obtaining a pathologic diagnosis of breast cancer. This approach allows definitive diagnosis prior to surgical intervention, which facilitates surgical planning. The core biopsy also provides information regarding tumor grade, type, and phenotype as currently defined by ER, PR, HER2, and Ki67. These biomarker studies guide adjuvant treatment, and may also be used to determine the likelihood of response to neoadjuvant systemic treatment, as surrogates for molecular classification or through the use of tools like the Magee Equation 3 [20]. The critical nature of these results on patient management necessitates excellent concordance between the biomarkers performed on the core biopsy and the surgical resection specimens. Our results generally support prior studies showing that there is acceptable concordance between core biopsies and resection specimens [6–16]. By entirely submitting the invasive carcinoma in the surgical resection specimens, we have also shown that the core biopsy reflects the tumor as a whole in the vast majority of cases. Although a significantly lower mean ER histologic score was observed in the surgical resections as compared with the core biopsy, this finding is unlikely to meaningfully impact treatment decisions in most cases. Core biopsy was falsely negative in 1 case for ER, and 3 cases for PR, in which weak expression was observed in the surgical resections. In our study, a statistically significant difference in mean Ki67 between the core biopsy and surgical resection was not observed. This finding further supports the notion that core biopsy assessment of Ki67 can be useful in determination of surrogate molecular phenotype. Chen, et al. observed good agreement between core biopsy and open excisional biopsy for Ki67 expression using a cut-off of 14%, which is often used in assessment of Luminal A vs. Luminal B phenotype [21, 22].

Although good concordance for HER2 status was observed between core biopsy and surgical resection, we observed 10/86 cases in which the core biopsy HER2 results were negative but areas of equivocal expression were observed on the resection specimen. In 9/10 cases, FISH studies performed on the area of strongest HER2

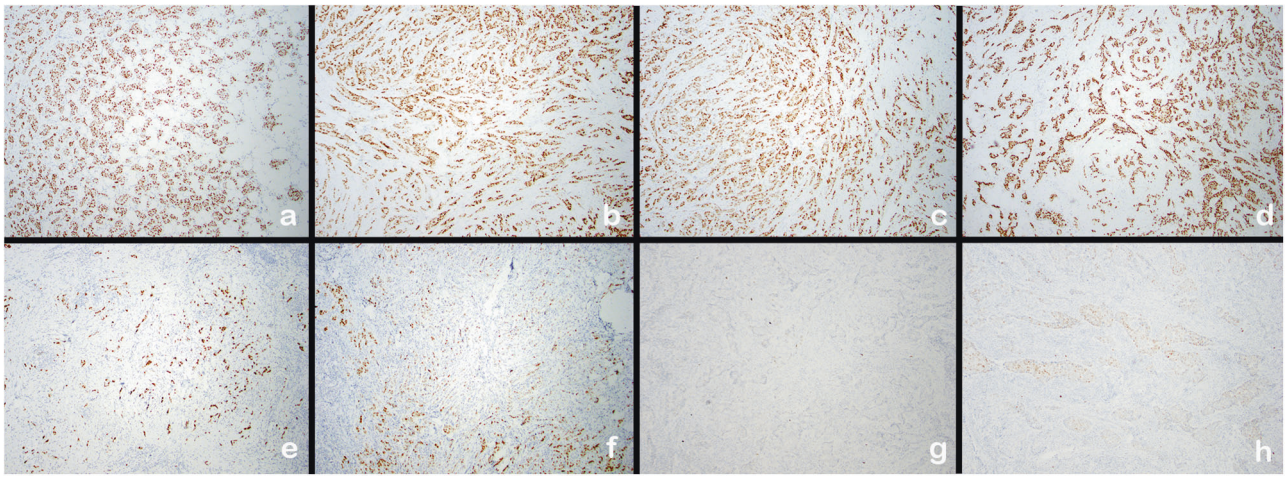

Fig. 4 Intratumoral variation of progesterone receptor (PR) immunohistochemical expression: **a**–**d** Case 19 (low variation)—individual tissue blocks with histologic scores of 120, 150, 150, and 160, respectively, (coefficient of variation 0.12, quartile coefficient of dispersion 0.03), **e**–**h** Case 86—individual tumor blocks with histologic scores of 105, 190, <1, and 53, respectively, with 4 of 8 submitted tumor blocks shown (coefficient of variation 0.96, quartile coefficient of dispersion 0.69)

membranous expression were negative for amplification by both *Her-2/neu*/cep17 ratio and absolute *Her-2/neu* gene copy number. One case had an equivocal result, with *Her-2/neu* gene copy number of 4.3, but ratio <2.0. These results suggest that although equivocal expression by HER2 immunohistochemistry may be observed on the surgical resection but not the core biopsy, *Her-2/neu* amplification by 2013 ASCO/CAP criteria is unlikely in these areas and the opportunity for targeted anti-HER2 therapy is unlikely to be missed. A large multi-institutional study of HER2 immunohistochemistry concordance between core biopsy and surgical resection specimens also noted that the majority of discordance was observed between negative (HER2 0/1+) and equivocal (HER2 2+) results [10].

Possible explanations for differences in biomarker status between core biopsy and surgical resection have included tumor heterogeneity, core biopsy sampling of the periphery of the tumor, and delayed fixation of surgical resection specimens [9, 23]. Increasing concordance has been reported with increasing numbers of tissue cores in the core biopsy specimens [12]. Advantages of the current study include adherence to ASCO/CAP guidelines for specimen processing (cold ischemic and formalin-fixation times) and comparison with the entire tumor.

In comparing biomarker expression patterns of invasive carcinoma and ductal carcinoma in situ, overall we observed similar ER and PR histologic scores in each component of the tumors. Near-perfect concordance was observed for ER between in situ and associated invasive carcinoma, while more variability was observed with PR.

Rates of discordance for ER and PR between ductal carcinoma in situ and invasive ductal carcinoma were previously studied by Steinman, et al. [24] who observed discordance more often in high-grade tumors, and also a consistent pattern of discordant expression, i.e., in which ductal carcinoma in situ was positive and invasive carcinoma was negative. Our cohort did include a few cases in which the in situ component was negative and the invasive carcinoma was positive. Ki67, interestingly, showed lower proliferative activity in in situ carcinoma as compared with invasive carcinoma.

Although no statistically significant difference in HER2 expression between ductal carcinoma in situ and invasive carcinoma was observed, 11 cases did show positive or equivocal expression in ductal carcinoma in situ but not in invasive carcinoma. Clinical significance of positive HER2 in ductal carcinoma in situ is unclear at present, but this finding underscores importance of scoring only invasive carcinoma, and further supports that it is not cost-effective to perform biomarker on core biopsies showing only ductal carcinoma in situ [25]. In select cases, immunohistochemical stains for myoepithelial markers, such as p63, may be needed to ensure scoring of the invasive component and for reflex FISH studies in equivocal cases. Occasionally presence of admixed ductal carcinoma in situ with differing HER2 expression than invasive cancer can significantly impact multi-gene assay test results.

Tumoral heterogeneity can be described and studied in a multitude of ways, including molecular, phenotypic, temporal, and spatial, and all must be considered in the pursuit of truly personalized cancer treatment. Intratumoral heterogeneity is incompletely understood, but is thought to arise by either the cancer stem cell hypothesis and/or the clonal evolution hypothesis. The cancer stem cell hypothesis attributes heterogeneity to a hierarchical organization of

Table 6 Progesterone receptor-negative "outlier" tissue blocks with corresponding estrogen receptor, Ki67 labeling index, and HER2 immunohistochemical score results

Case number	Core biopsy PR *H*-score	Individual tumor blocks PR *H*-score	ER *H*-score	Ki67 labeling index	HER2 score
3	50	27	300	5	0
		0	300	5	0
		18	280	10	1
		0	270	10	1
		55	270	5	1
6	22	0	195	10	1
		0	180	8	1
		30	170	20	0
15	20	115	195	25	1
		63	165	45	2
		50	180	20	2
		0	100	35	2
23	15	0	250	30	2
		0	260	30	2
		55	230	20	2
25	0	1	0	85	1
		0	0	85	1
		0	0	85	1
		0	0	80	1
		0	0	85	1
		13	0	75	1
46	110	80	260	1	1
		90	190	1	0
		0	220	1	0
60	60	90	250	40	2
		150	265	30	2
		0	290	30	1
		140	290	30	2
65	60	40	180	50	3
		60	200	50	3
		0	180	50	3
		0	210	50	3
95	80	20	290	20	1
		40	290	15	1
		80	290	20	1
		0	290	15	1
86	0	190	290	20	1
		105	290	30	2
		0	205	20	2
		70	280	30	2
		23	280	30	2
		53	280	15	2
		90	290	30	2
		0	295	20	1

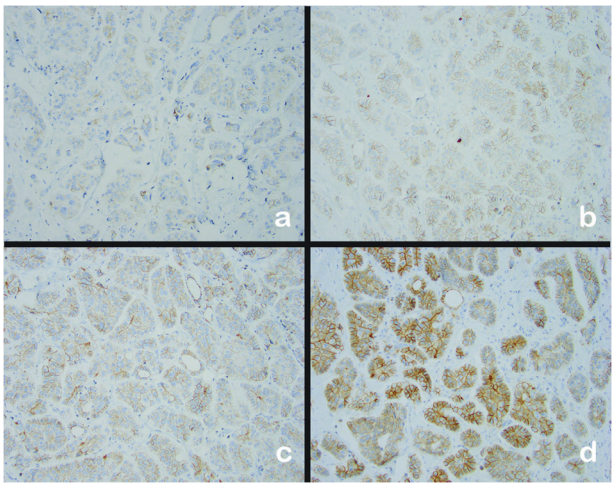

Fig. 5 Case 29—Heterogeneity of HER2 Immunohistochemistry in individual tumor blocks **a** HER2 1+, **b** HER2 2+, **c** HER2 2+, and **d** HER2 3+ with 4 of 5 submitted tumor blocks shown

cancer cells, in which a small population of self-renewing tumorigenic cells grows and differentiates [26].

In the clonal evolution model, cancer cells acquire genetic aberrations during tumorigenesis and tumor evolution, leading to subpopulations that harbor both founder mutations but also subsets of genetic abnormalities that may be reflected in phenotypic changes [27, 28]. Spatial phenotypic heterogeneity of common biomarkers presents challenges due to the trend toward smaller diagnostic biopsy samples (core biopsies), combined with the need for accurate characterization of biomarkers for targeted treatments. Many studies have focused on differences between primary tumor phenotype and the phenotype of recurrences and metastases (temporal heterogeneity) [29–32]. These changes form the basis for the recommendation that repeat biomarker studies should be performed tissue biopsies from recurrences and metastases. Without characterization of the entire tumor at the time of diagnosis, which is impractical, the attribution of differences in tumor phenotype over time to either heterogeneity of the primary tumor or selective pressure from treatment effects is difficult.

One goal of this study was to assess for spatial phenotypic heterogeneity of commonly used biomarkers in breast

cancer through immunohistochemical testing of the entire tumor and statistical analysis of dispersion. In comparing tumor blocks, this study addressed regional intratumoral heterogeneity but not intermingled cells with different phenotypes.

Analysis of interblock variation showed greater coefficient of variation and quartile coefficient of dispersion for PR than for ER, with mean coefficient of variation and quartile coefficient of dispersion 3.25 and 3.5× greater in the invasive carcinoma, respectively. Due to its regulation by ER, PR has been described as an indicator of an intact ER pathway [33]. PR negative breast carcinomas have a worse prognosis, and low or absent PR expression are often used as a surrogate for Luminal B type breast carcinomas [34–39]. While unlikely to have major clinical consequences, the heterogeneity of semiquantitative PR expression by histologic score reported here could be of interest in selection of blocks for genomic assays and in use of tools such as IHC4 and the Magee Equations™. Overall, however, for ER and PR, the degree of variation observed suggests that for initial treatment decisions, biomarker testing of either the core biopsy or one tumor block will be sufficient in the majority of cases. In rare cases, however, a single tumor block may not be reflective of intensity of ER or PR expression of the tumor as a whole, and certainly raises the possibility that spatial heterogeneity may be responsible for some cases in which the tumor phenotype of recurrences and metastases is different from the primary tumor if the core biopsy or one tumor block has been tested. Use of tumor morphology (grade, special types, or morphologic heterogeneity) may help to guide pathologists in determining whether repeat testing on the resection specimen or on multiple tissue blocks could be considered.

Assessment of Ki67 labeling index in invasive breast carcinomas has been established as important for prognosis, prediction of chemotherapy benefit, and assignment of surrogate molecular subtype [40, 41]. Overall mean Ki67 in surgical resection was not significantly different from core biopsy results; however, differences were observed in individual tumor blocks as compared to the core biopsy in 24% of cases when using a cut-off of 25% [19]. These results suggest using Ki67 as part of a multivariable model for prediction of chemotherapy benefit rather than as a stand-alone marker. We have observed coefficient of variation and quartile coefficient of dispersion for Ki67 in whole tumors to be intermediate between dispersion for ER and PR expression by histologic score, and variation in individual tumors to be up to 35% when hotspots are included. In a recent study of cold, intermediate, and hotspots, Focke et al. [42] showed that the coefficient of variance between the spots was higher in ER-positive tumors than in ER-negative tumors. The nested analysis of variance indicated that in both ER-positive and ER-negative tumors, variance in Ki67 labeling index within tumors contributed more to the total variance than the variance between tumors [42].

We did not observe increased heterogeneity when cases were grouped by size (T stage), although most of the tumors evaluated were T1c or T2 tumors. Only 1 T3 tumor was included in this study, and therefore these findings may not apply to larger or locally advanced breast cancers.

Testing for *Her-2/neu* amplification/HER2 overexpression is necessary for new diagnoses of invasive breast carcinoma, as well as recurrences and metastatic foci, if tissue is available. This testing is generally accomplished through immunohistochemistry with reflex FISH for equivocal results, or FISH alone. Criteria for positive, negative, and equivocal results have changed over time, and the most recent ASCO/CAP guidelines utilize a 10% cut-off for strong, complete membranous expression as the cut-off between positive and negative by immunohistochemistry, while less intense expression would be considered as equivocal or negative [17]. For pathologists charged with determining HER2 status either by immunohistochemistry or FISH, the worry of missing highly HER2-amplified foci can lead to identification of complex low-level amplification and equivocal results of uncertain clinical significance. Furthermore, outcome data is limited in patients with heterogeneous patterns of HER2 expression. In one study by Lee, et al, tumors with <75% HER2 3+ expression was associated with less objective response to trastuzumab and shorter time to progression than HER2-positive tumors with >75% cells with HER2 3+ expression [43]. The pattern of expression in which a minority of the tumor showed positive HER2 by immunohistochemical expression, while the remainder was negative or equivocal, was uncommon in the current study. A smaller number of HER2-positive patients than would be expected based on incidence of HER2-positive cancer was observed, however, possibly due to exclusion of cases with neoadjuvant systemic therapy.

This comprehensive biomarker analysis of 100 cases of invasive breast carcinoma shows that commonly used biomarker studies performed on core biopsies are reflective of the entire tumor in the vast majority of cases. Significant differences in biomarkers between ductal carcinoma in situ and invasive carcinoma were only observed for Ki67, while ER, PR, and HER2 were generally concordant, consistent with the role of ductal carcinoma in situ as a precursor to associated invasive carcinoma. Intratumoral heterogeneity in invasive carcinoma is greater for PR than ER, but T2 tumors did not have greater heterogeneity than T1 tumors in the cases studied.

These findings support the continued use of current protocols for biomarker assessment in primary, recurrent, and metastatic invasive breast carcinoma.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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