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Breast Cancer Biomarker Changes after Neoadjuvant Chemotherapy: A Single Institution Experience and Literature Review

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Abstract

Chemotherapy and hormonal treatment decisions for breast cancer are influenced by the expression of tumor biomarkers, including estrogen receptor, progesterone receptor and human epidermal growth hormone receptor 2 (HER2/*neu*). These biomarkers are most commonly assessed by immunohistochemical staining and fluorescence in situ hybridization studies performed on breast cancer biopsy specimens. Biomarker studies performed on excisional specimens may be discordant with those performed on core needle biopsy, especially after neoadjuvant chemotherapy. We evaluated 195 cases at our institution from 2002 to 2015 to determine the effects of neoadjuvant chemotherapy on the expression of the aforementioned biomarkers and Ki-67. Forty-nine (25.1%) cases showed complete pathologic response after neoadjuvant chemotherapy. Twelve cases (8.6%) showed a change in estrogen receptor status after neoadjuvant chemotherapy. Twenty-four cases (17.1%) showed a change in progesterone receptor status, predominantly from positive to negative (p = 0.0002). HER2/*neu* expression was evaluated by both immunohistochemical staining and fluorescence in situ hybridization, where two (1.5%) (p = 1.000) and eight (8.2%) (p = 0.4795) cases changed expression status, excluding equivocal cases, respectively. Ki-67 was also evaluated, with 49 cases (48%) (p <0.0001) showing altered proliferation indices after neoadjuvant therapy. The differences were statistically significant for progesterone receptor and Ki-67. Overall survival was also evaluated, and showed statistically significant differences between groups that changed progesterone receptor status after neoadjuvant therapy. Given the therapeutic implications of altered biomarker status after neoadjuvant chemotherapy, we recommend repeating biomarker studies on resection specimens to most appropriately guide adjuvant chemotherapy decisions.

Keywords: Breast cancer; Biomarker changes; Neoadjuvant chemotherapy

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Introduction

Breast cancer is the most common cancer affecting women in the United States. Neoadjuvant chemotherapy decisions for breast cancer are highly influenced by the expression of tumor biomarkers, including Estrogen Receptor (ER), Progesterone Receptor (PR), and human epidermal growth hormone receptor 2 (HER2/*neu*), that are performed on needle core biopsy specimens through immunohistochemical (IHC) staining or Fluorescence In Situ Hybridization (FISH) studies. While neoadjuvant chemotherapy for the treatment of breast cancer was historically reserved for patients with locally advanced, inoperable disease, neoadjuvant therapy is now being increasingly used for the initial treatment of smaller and operable tumors to downstage and perform conservative surgical excision [1,2]. After neoadjuvant chemotherapy and surgery, biomarker studies are frequently repeated on resection specimens, and may be different from biomarker studies performed on core needle biopsies. This finding may represent a variety of reasons, such as chemotherapy effect or intratumoral heterogeneity, a well-documented characteristic of breast carcinomas. Previous studies evaluating the effect of neoadjuvant chemotherapy on tumor biomarkers have not been entirely in agreement [3-5], and this information may affect treatment decisions following surgery. This study aims to add to the growing body of literature the effects of neoadjuvant chemotherapy on the expression of ER, PR, HER2/*neu* by IHC and FISH, and Ki-67, and includes clinical follow-up for our cohort, which most of the current literature did not report. We also present the most comprehensive literature review to date, and conclude with a summary of our statistically significant findings with recommendations.

Materials and Methods

Ethics statement

This project was conducted with the approval of the University of California, Los Angeles (UCLA) Institutional Review Board.

Study group

A retrospective chart review of our institution's pathology database was conducted to identify patients with invasive breast carcinoma who underwent neoadjuvant chemotherapy prior to surgical resection between 2002–2015. Patients who had both pre- and post-treatment pathology reports with biomarker studies available were included. Male patients and patients with multifocal, bilateral, and recurrent disease were excluded. Clinical and pathological data were collected via review of medical records and extracted to the Research Electronic Data Capture (REDCap) [6] database. Clinicopathologic data collected included patient age at the time of surgery, follow-up data, and tumor characteristics, such as histologic type, grade, and ER, PR, HER2/*neu*, and Ki-67 status. The modified Bloom-Richardson scoring system was used to determine histologic grade.

Immunohistochemical (IHC) methods and evaluation

Formalin-fixed, paraffin-embedded human breast cancer tissue specimens from 195 patients with invasive breast carcinoma were used in the study. IHC stains were performed with ER and PR antibody clones with appropriate positive and negative controls. Slides were baked for 1-4 hours in a 60°C oven and then deparaffinized in xylene and brought into tap water through graded alcohols (100% x4, 95%x2). Slides were then treated with 0.5% hydrogen peroxide in absolute methyl alcohol for 10 minutes to quench endogenous peroxidase activity, and then washed with tap water and deionized water. Heat-induced antigen retrieval was then performed by placing slides in 0.01 M citrate buffer, pH 6.0 preheated to 95°C in a vegetable steamer (Black & Decker); heat-treatment lasted for 25 minutes, followed by a 15-minute room temperature cool-down period. Following rinse in PBS, slides were immunostained on a DAKO Autostainer, using a goat anti-mouse Poly-HRP secondary antibody (MACH2 Mouse HRP-Polymer, Biocare Medical, Concord, CA) as the detection system. The staining procedure involved a 45minute primary antibody incubation with mouse monoclonal antibodies against Estrogen Receptor clone SP1 (1/50 dilution, Biocare Medical, Concord, California) and Progesterone Receptor clone 636 (1/300 dilution, DAKO Corp, Carpenteria, CA), followed by a PBS wash and a 30 minute incubation in MACH2 mouse HRP-Polymer. The negative control reagent was mouse IgG1 or diluent alone. Diaminobenzidine was used as the chromogen to effect visualization of the peroxidase enzyme. Slides were then counterstained with Harris hematoxylin. Using appropriate positive and negative controls, the test for the presence of these hormone receptor proteins was performed by the immunoperoxidase method, and reported according to the 2009 CAP-ASCO Guidelines for Hormone Receptor testing. Tissues were fixed between 6-72 hours in 10% neutral buffered formalin. A positive ER or PR tumor showed greater than or equal to 1 percent of cells staining, and results were semi-quantitated with percentage and intensity of positive tumor cells.

For Ki-67, clone MIB-1 (Mouse Monoclonal, DAKO) was diluted 1:50 in buffered Calcium Chloride (0.074 gm Calcium Chloride dihydrate in 50 ml of 0.05M Tris-buffered saline, pH 7.4, containing 25ul of Tween 20). Percentage of cells positive for Ki-67 was evaluated, and the Ki-67 index was then categorized into a three-grade system: <10% (low), 10-20% (intermediate), and ≥20% (high).

Both IHC analysis for HER2 protein and fluorescence in-situ hybridization for *ERBB2* gene were performed on all specimens from our facility at the David Geffen University of California at Los Angeles (UCLA) Medical Center from years 2002 through 2015. Optimal tissue handling requirements (e.g. time to fixation) were followed and recorded, particularly after publication of the American Society of Clinical Oncology/College of American Pathologists guidelines, after 2007.

The US Food and Drug Administration FDA-approved HercepTest™ was performed using DAKO A0485 polyclonal antibody kit (DAKO Corp, Carpenteria, CA). The results were scored by US Food and Drug Administration (US FDA) guidelines prior to publication of the American Society of Clinical Oncology/College of American Pathologists guidelines, with immunohistochemistry scoring of 3+ cases staining >10% of tumor cells.

Fluorescence in-situ hybridization analysis

Fluorescence in-situ hybridization (FISH) was performed using the US Food and Drug Administration (FDA)-approved PathVysion™ *HER2* DNA Probe Kit (PathVysion™ Kit), which is designed to detect amplification of the *ERBB2* gene via fluorescence in-situ hybridization in formalin-fixed, paraffin-embedded human breast cancer tissue specimens. Fluorescence in-situ hybridization analysis with VYSIS dual-color probes specific for chromosome 17 centromere and the *ERBB2* gene (17q11.2) was performed and examined by two independent technologists. Slides containing 4-micron sections were submitted for fluorescence in-situ hybridization analysis. For each slide, based on the corresponding hematoxylin & eosin slide, the invasive tumor area(s) were circled with a secure line marker. Areas containing ductal carcinoma in-situ or normal tissue were excluded from fluorescence in-situ hybridization testing. Slides were baked overnight at 60°C and pretreated using the VP2000 tissue processor as per manufacturer's protocol (Abbott Molecular, Abbott Park, IL). Amplification of the *ERBB2* gene was detected by using the PathVysion™ Kit; the instructions in the package insert were followed for the hybridization, post-hybridization washing, and analysis steps (Abbott Molecular, Abbott Park, IL). All cases were scored according to US Food and Drug Administration guidelines for HER2 immunohistochemistry and used *ERBB2* gene fluorescence in-situ hybridization amplification cutoff value of 2.0 before 2007 and after 2013. Between 2008 – 2012, FISH and HER2 IHC cutoffs were used from ASCO/CAP guidelines.

Statistical analysis

Results for the modified Bloom Richardson scoring parameters, as well as ER, PR, HER2/*neu* statuses and Ki-67 levels were evaluated with the Wilcoxon-signed rank test. Univariate survival analysis was performed using log rank tests and visualized using Kaplan-Meier survival curves. Overall survival was defined as the interval from the date of resection to the date of death from any cause, or date of last follow-up after known or suspected hospice. Patients were otherwise censored at the date of last follow-up. Stata 12.0 software (Stata Corporation, College Station, TX, USA) was used for all statistical analyses. A p-value of <0.05 was considered statistically significant.

Literature review

A comprehensive search of the existing published literature between 1991-2016 in PubMed was performed. MeSH terms included

Table 1: Pre-treatment Patient and Tumor Characteris.

Age (years)	
<50	100 (51)
>= 50	95 (49)
Histologic type	
Ductal	176 (90)
Lobular	8 (4)
Other	11 (6)
Histologic grade	
I	14 (7)
II	66 (34)
III	105 (54)
Not reported	10 (5)
ER status	
Positive	121 (62)
Negative	72 (37)
Not reported	2 (1)
PR status	
Positive	99 (50)
Negative	95 (49)
Not reported	1 (1)
HER2 FISH status	
Positive	63 (32)
Negative	88 (45)
Not performed	44 (23)
Ki67	
<10% (low)	8 (4)
10-20% (intermediate)	14 (7)
>=20% (high)	122 (62)
Not reported	52 (27)

Table 2: Comparison of Mean Modified Bloom-Richardson Scores Pre- and Post-Neoadjuvant Chemotherapy (n=89).

	Pre-therapy	Post-therapy	*p-value*
Tubule formation	2.84	2.85	0.76
Nuclear pleomorphism	2.51	2.56	0.26
Mitotic activity	2.00	1.79	0.005
m-BRS score	7.33	7.20	0.19

neoadjuvant, chemotherapy, breast, biomarker, ER, PR, and HER2. The listed references in relevant articles retrieved were also reviewed. Articles that met our criteria for review were included only if they were published in the English language and were accessible online.

Results

Clinical and pathological data for 195 cases that fulfilled the inclusion criteria were collected (Table 1). The median age at the time of diagnosis was 49 (interquartile range, 41-58). All patients were female. The majority of tumors were of invasive ductal carcinoma, no specific type (90%), while the remaining were of invasive lobular (4%) or other histologic types (6%). There were 14 (7%) Grade I tumors, 66 (34%) Grade II tumors, and 105 (54%) Grade III tumors, and the histologic grade for 10 (5%) tumors was not reported. Prior

Table 3: Biomarker status change after Neoadjuvant chemotherapy.

	ER	PR	Her2 IHC	Her2 FISH
Available cases	140	140	130	97
Positive to negative	6	21	1	5
Negative to positive	6	3	1	3
Total changes (%)	12 (8.6)	24 (17.1)	2 (1.5)	8 (8.2)
p-value	1.000	0.0002	1.000	0.4795

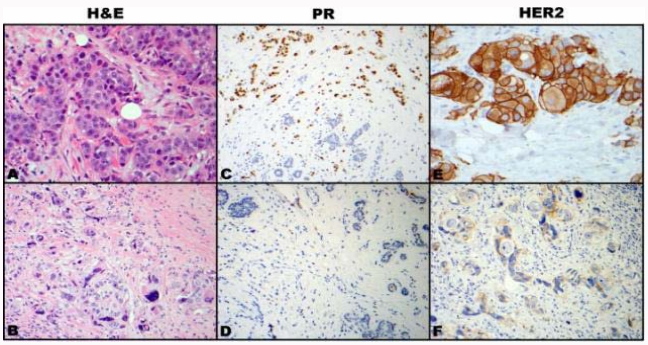

Figure 1: A-B. After neoadjuvant chemotherapy, invasive ductal carcinoma shows marked treatment related changes, including fibrosis and pleomorphism (B), as compared to untreated tumor (A) (H&E, original magnification x200).
C-D. Twenty-one tumors that were initially positive for PR (C), lost expression of PR after neoadjuvant chemotherapy (D) original magnification x400). **E-F.** One tumor that was initially HER2 positive by IHC (E) lost expression of HER2 after neoadjuvant chemotherapy (F) (original magnification x400).

Table 4: Ki67 Levels Pre- and Post-Neoadjuvant Chemotherapy.

	Post-Treatment Ki67			
Pretreatment Ki67	Low (<10%)	Intermediate (10-20%)	High (≥ 20%)	Total
Low (<10%)	6	1	0	7
Intermediate (10-20%)	8	1	3	12
High (≥ 20%)	25	12	46	83
Total	39	14	49	102

to neoadjuvant chemotherapy, most of tumors largely demonstrated high proliferation indices based on Ki-67 staining of over 20% of tumor cells (62%), while the remaining were either of low (<10% staining of tumor cells) proliferation index (4%), intermediate (10-20% staining of tumor cells) proliferation index (7%), or not reported.

Following neoadjuvant chemotherapy, 49 patients (25.1%) showed pathologic complete response with no residual invasive carcinoma identified after extensive sampling of resection specimens, and four patients (2.1%) had only minimal or microinvasive carcinoma. The changes to the modified Bloom-Richardson scores (m-BRS) were evaluated for 89 patients (45.6%) with available data (Table 2). The pre-therapy mean tubule formation score was 2.84, while the post-therapy mean was 2.85 (p = 0.76). The pre-therapy mean nuclear pleomorphism score was 2.51, while the post-therapy mean was 2.56 (p = 0.26). The pre-therapy mean mitotic activity score was 2.00, while the post-therapy mean was 1.79 (p = 0.005). A significant decrease in mitotic rate was observed after neoadjuvant chemotherapy. The pre-therapy mean total m-BRS score was 7.33, while the post-therapy mean was 7.20 (p = 0.19). Downgrading of m-BRS score occurred with 29 patients. (Figure 1) shows invasive ductal carcinoma pre- (A) and post- (B) neoadjuvant chemotherapy.

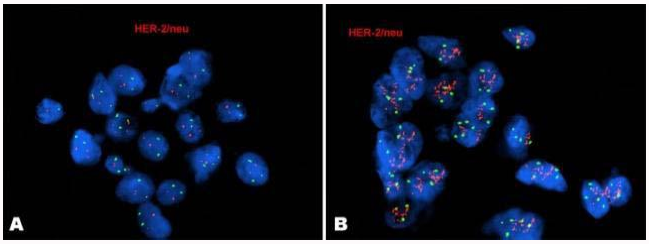

Figure 2: Three tumors that were initially HER2 negative by FISH (A) were found to be *ERBB2* gene amplified by FISH after neoadjuvant chemotherapy (B).

Changes in hormonal receptor expression

Pre and post neoadjuvant treatment ER and PR statuses were available for 140 patients (Table 3). Of these, ninety-six (69%) tumors were initially ER positive, six (6%) of which became negative following neoadjuvant therapy. Likewise, six (4%) initially ER-negative tumors became positive post-treatment. The change in ER status was not statistically significant. Eighty-two tumors (59%) were initially PR positive, and 21 (26%) of these became PR negative after neoadjuvant therapy (Figure 1C and D). Three (2%) initially PR-negative tumors became positive for PR post-treatment. Overall, the change in PR status was statistically significant ($p = 0.0002$).

Changes in HER2/*neu* IHC and FISH

HER2/*neu* status was evaluated both by IHC and FISH (Table 3). HER2/*neu* IHC data pre- and post-treatment was available for 130 tumors. Of these, 2 tumors (1.5%) had a change in status after treatment, including one (0.8%) that changed from negative to positive, and one (0.8%) that changed from positive to negative (Figure 1E and F). Data on HER2/*neu* FISH status was available for 97 patients, and eight (8.2%) changed statuses after neoadjuvant therapy. These included five (5.2%) that changed from positive to negative and three (3.1%) that changed from negative to positive (Figure 2). Neither the change in HER2/*neu* expression by IHC ($p = 1.000$) nor by FISH (p=0.4795) was statistically significant.

Changes in Ki-67 expression

The majority of tumors demonstrated high Ki-67 proliferation indices (Table 4). Pre- and post-treatment data on Ki-67 proliferation indices was available for 102 tumors, 83 (81.3%) of which had high proliferation indices. Twelve (14.4%) of these changed to intermediate proliferation index, and 25 (30.1%) changed to low proliferation index. Overall, 49 (48.0%) changed Ki-67 proliferation indices after neoadjuvant therapy, showing a statistically significant change (p <0.0001).

Biomarker changes and patient outcome

At the time of last follow-up, there were 18 (9%) deaths or patients in hospice care. Those patients in hospice care were considered dead of disease for our analysis since there were no subsequent medical follow-up records. Median follow-up was 36.0 months (interquartile range, 18.4 to 70.1). (Tables 5 and 6) detail patient outcomes by hormone receptors and HER2 phenotype, as determined on biopsy and excision specimens, respectively. Patients were also grouped by biopsy and excision biomarker statuses, and overall survival was assessed between groups. (Table 7) lists the number of deaths in each group, with differences assessed by logrank analyses. Statistical significance was seen only with PR groups ($p = 0.0319$), as visualized by Kaplan-Meier curves (Figure 3).

Table 5: Outcome by biopsy phenotype.

	Dead	Alive
ER-PR-HER2-	5	33
ER+PR+HER2+	3	28
ER+PR+HER2-	3	55
ER+PR-HER2-	2	8
ER-PR+HER2-	0	3
ER+PR-HER2+	2	13
ER-PR+HER2+	0	3
ER-PR-HER2+	2	27
Total	17	170

Table 6: Outcome by excision specimen phenotype.

	Dead	Alive
ER-PR-HER2-	4	24
ER+PR+HER2+	2	17
ER+PR+HER2-	1	44
ER+PR-HER2-	6	16
ER-PR+HER2-	0	1
ER+PR-HER2+	2	9
ER-PR-HER2+	2	14
Total	17	125

Table 7: Number of deaths by biomarker conversion.

	ER	PR	Her2 IHC	Her2 FISH
Available cases	140	140	130	97
Negative to negative	6	11	7	9
Positive to positive	10	3	2	3
Negative to positive	1	0	0	0
Positive to negative	0	3	0	1
Equivocal cases			6	
p-value	0.5383	0.0319	0.9943	0.7393

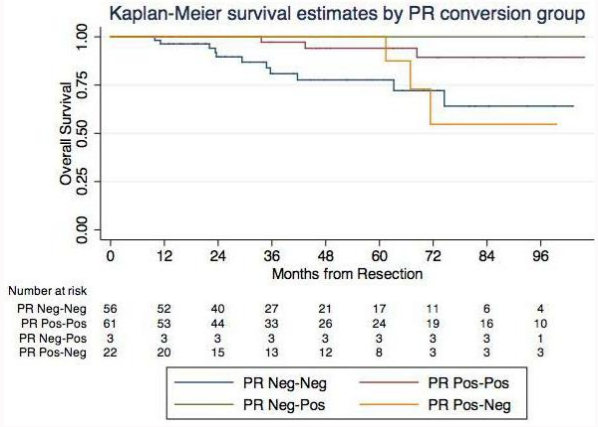

Figure 3: Kaplan-Meier survival analysis showed significant differences in overall survival when grouped by biopsy and excision PR status ($p = 0.0319$).

Literature review

We performed a comprehensive literature review of all studies evaluating biomarker change after neoadjuvant chemotherapy in breast cancer (Table 8). Thirty-one articles were identified. Reports of

Table 8: Literature Review.

	Year	Author	No.	ER	PR	HR changes	HR (+ to -)	HR (- to +)	HER2 changes	HER2 (+ to -)	HER2 (- to +)	Ki-67
1	2016	Gahlaut R et al. [30]	133	16/133 (12%)	18 (14.5%)				8/133 (7.1%)			
2	2016	Lim SK et al. [38]	290			17.9%	23 (10.8%)	29 (37.2%)	17 (20.7%)	17 (20.7%)	0	
3	2015	Jin X et al. [34]	423			18.4%	55 (13%)	23 (5.4%)	40 (9.5%)	27 (6.4%)	13 (3.1%)	
4	2015	Jin G et al. [3]	138	31 (22.5%)	31 (22.5%)				21 (15.2%)			71 (51.5%)
5	2015	Zhou X et al. [32]	107	15 (14%)	26 (24.3%)				5 (4.7%)			
6	2015	Avci N et al. [39]	100						13 (13%)			41 (41%)
7	2015	Ge WK et al. [40]	95	12.70%	21.10%				5 (10%)			38.00%
8	2014	Basak et al. [41]	90	10 (12.5%)	17 (21.2%)							
9	2014	Tsai YM et al. [42]	78	16 (20%)	18 (23%)				27 (35%)			
10	2014	Montagna E et al. [43]	904	5%	67%							40%
11	2014	Tan QX et al. [37]	267			15.7%						
12	2013	Lee HC et al. [44]	120	15/120 (12.5%)	22/120 (18.3%)				24/107 (22.4%)			
13	2013	Cockburn A et al. [45]	172	9.02%	18.40%				27 (20%)			7.00%
14	2013	Yang YF et al. [46]	113	14/113 (12.4%)	18/113 (15.9)	18/113 (15.9%)			17/113 (15.0%)			
15	2013	Dede DS et al. [47]	63	2 (5.7%)	10 (15.8%)							
16	2013	Valent A et al. [48]	344						30 (9%)			
17	2012	Kinsella MD et al. [49]		0								
18	2012	Pachnicki JP et al. [50]	47	26.60%	33.30%							
19	2011	Zheng S et al. [51]	209	75/177 (42.4%)	98/177 (55.4%)				(46/173) 26.6%			
20	2011	Kumaki N et al. [52]	53	5 (9.4%)	7 (13.2%)				5/49 (10.2%)			49 (92.4%)
21	2009	Hirata T et al. [36]	368	55/368 (15%)	107/368 (29%)							
22	2008	Kasami M et al. [53]	173	19 (11.0%)	27 (15.6%)				0			
23	2008	Neubauer H et al. [4]	87	7/87 (8%)	16/87 (18.4%)					11/86 (13%)	2/86 (2%)	
24	2008	Adams AL et al. [54]	26	2 (7.7%)	6 (23%)							
25	2005	Varga Z et al. [55]	23			7 (30%)			8 (35%) IHC 2 (13%) FISH			
26	2003	Taucher S et al. [56]	191	27/191 (14%)	41/191 (21%)							
27	2003	Faneyte IF et al. [57]	50	14/49 (29%)						2/50 (4%)	1/50 (2%)	19/50 (38%)
28	2003	Lee SH et al. [58]	56			3 (5%)						
29	1996	Jain V et al. [59]	18	3/18 (16.6%)	4/18 (22.2%)							
30	1994	Lo SS et al. [60]	10	2/10 (20%)	3/10 (30%)							
31	1991	Morris DM et al. [61]	20			2/20 (10%)						
	UCLA		140	12 (8.6%)	24 (17.1%)				2 (1.5%) IHC 8 (8.2%) FISH			49 (48.0%)
	Total		**5,304**	**0%-42.4%**	**13.2%-67%**	**5%-30%**			**0%-5%**			**7%-92.4%**

ER changes ranged from 0%-42.4% and reports of PR changes ranged from 13.2%-67%. A number of studies evaluated hormone receptor changes together, and reported results that ranged from 5-30%. HER2 changes, either by IHC or FISH, ranged from 0% - 35%. Lastly, changes in Ki-67 proliferation index ranged from 7% to 92.4%.

Summary of our results

Twelve cases (8.6%) showed change in ER status after neoadjuvant

chemotherapy. Twenty-four cases (17.1%) showed change in PR status, predominantly from positive to negative (p = 0.0002). HER2/*neu* expression was evaluated by both IHC and FISH, where 35 cases changed expression status by IHC (p=0.0130), and eight by FISH (p = 0.4795). Excluding equivocal cases by both IHC and FISH testing, only two cases (1.5%) by IHC and eight cases (8.2%) by FISH showed alterations in HER2/*neu* status. Ki-67 was also evaluated, with 49 cases (48%) (p <0.0001) showing decreases in proliferation indices after neoadjuvant therapy. In addition, overall survival was assessed between groups based on pre- and post-neoadjuvant chemotherapy biomarker statuses, and showed statistically significant differences between PR groups (p = 0.0319).

Conclusion

Recently, the American Society of Clinical Oncology (ASCO) published clinical practice guidelines recommending re-biopsy of breast cancer metastases to re-evaluate ER, PR and HER2/*neu* expression. However, evidence is still lacking on whether changes to chemotherapy regimens should be made on the basis of altered biomarkers in the adjuvant therapy setting. The Panel consensus was to use biomarker testing from metastatic tumors to direct therapy accordingly [7]. There are no ASCO guidelines on whether excisional biopsy specimens should be re-tested after neoadjuvant therapy, and if there are changes, whether adjuvant chemotherapy should be altered. Hence, the practice differs worldwide. Neoadjuvant chemotherapy is being increasingly used prior to surgical resection of breast cancer, with treatment regimens guided by hormone receptor status and HER2/*neu* expression from core needle biopsy samples. Previous studies have shown that biomarker expression may change after neoadjuvant chemotherapy in breast cancer. Several possibilities may explain the discordance between hormone receptors and HER2/*neu* expression and *ERBB2* gene amplification before and after neoadjuvant chemotherapy. Possible explanations include: 1. Discordances between core needle biopsy and excisional specimens due to sampling variability, 2. Intratumoral heterogeneity, 3. Interlaboratory variability, 4. Differences due to ER and PR cutoff changes from 10% previously to >1% currently, as well as ASCO/CAP HER2/*neu* guideline changes, and 5. Instability of tumor biomarkers throughout tumor progression and with multiple relapses [8].

Discordances between core needle biopsy and excision due to sampling variability

Previous studies evaluating biomarker concordance have ranged from 0% to 42.4% for ER, 13.2% to 67% for PR, and 0% to 35% for HER2/*neu* [9,10]. In general, there is excellent agreement between biomarkers assessed in core needle biopsy and excisional specimens. Chen et al. [11] reported high concordance with ER, PR, and HER2 at 93.6%, 85.9%, and 96.3%, respectively [11]. Another study showed an almost perfect concordance for ER (k = 0.89; 95% CI: 0.65-1.0) and a substantial concordance for PR (k = 0.70; 95% CI: 0.46-0.93), HER2 (k = 0.61; 95% CI: 0.38-0.84) and Ki-67 (k = 0.74; 95% CI: 0.58-0.98) obtained between core needle biopsy and excision [12]. We also previously studied the reliability of core needle biopsy samples on breast cancer when compared to excisional specimens and reported high concordance rates for ER and PR, at 95% and 89%, respectively, and HER2/*neu* by IHC at 96% and by FISH at 100% [13].

Intratumoral heterogeneity

Somewhat related to issue #1 is intratumoral heterogeneity, which has also been well-documented in breast cancer. Discordances between pre- and post-treatment biomarker testing may represent sampling variability during initial biopsy, though marked intratumoral heterogeneity is rare [14,15]. Intratumoral heterogeneity has been proposed to arise from genetic alterations during the clonal evolution of tumor cells, that may subsequently result in genetic subclones within a single tumor [14]. Even without neoadjuvant chemotherapy, alterations of biomarkers have been reported between primary breast cancer compared with synchronous axillary metastasis or recurrent metastatic breast cancer [16]. Most of the literature have studied HER2 rather than hormonal receptors in regards to intratumoral heterogeneity. A study by Kurozumi et al. [17], evaluated HER2 negative breast cancer cases with IHC scores of 0 and 1+ and showed HER2 heterogeneity in 17 % of cases, using a more sensitive HER2 gene-protein assay. These patients were found to have a poor prognosis [17]. In particular, low histologic grade and hormone receptor positive tumors have been shown to have HER2 regional and genetic heterogeneity in 6.2% and 6.8% of cases, respectively [18]. *ERBB2* gene amplification genetic heterogeneity has also been reported and ranges from 5% to 30% in the literature [19].

Others, however, have shown intratumoral heterogeneity is not a significant factor. The largest translational study conducted to investigate the correlation of central IHC/FISH assessments with microarray mRNA readouts of ER, PR, and HER2 status is the Microarray In Node-negative and 1 to 3 positive lymph node Disease may Avoid ChemoTherapy (MINDACT) trial, which showed no support for intratumoral heterogeneity for the cause of biomarker discordance. MINDACT is an EORTC 10041 (BIG3-04), international, prospective, randomized, phase III trial with 6694 patients investigating the clinical utility of MammaPrint in selecting patients with early breast cancer for adjuvant chemotherapy [20].

Interlaboratory variability

Interlaboratory variability in hormone receptors and HER2/*neu* testing have been reported. In early 2000, Rhodes et al. [21], published data from 200 laboratories in the UK quality assessment for ER IHC testing and showed a considerable interlaboratory variability in low ER positive samples with a false negative rate of between 30% and 60%. High ER samples had over 80% concordance rate.

For HER2/*neu*, a prospective, randomized, three-arm, phase III trial was conducted by the Breast Intergroup (N9831) and found a poor concordance (74%) between local and central testing for HER2 status [22].The MINDACT trial study showed that ER had a positive agreement of 98% and a negative agreement of 95%, PR had a positive agreement of 85% and a negative agreement of 94%, and HER2 had a positive agreement of 72% and a negative agreement of 99% with central pathology [20]. In the current study, 41% of pretreatment biomarker testing was performed on core biopsies from outside institutions that were not repeated at our institution. Accordingly, this may play a factor in the discordant rate between pre- and post neoadjuvant chemotherapy specimens.

Differences due to ER and PR cutoff changes from 10% previously to >1% currently, as well as ASCO/CAP HER2/*neu* guideline changes

A change in cutoff valuesfor ER and PR may have resulted in discordancese between pre and post neoadjuvant chemotherapy hormonal receptor status. In 2010, the American Society of Clinical Oncology (ASCO) and College of American Pathologists (CAP) issued guidelines that tumors with ≥1% cells staining positive for

ER should be considered ER positive [23]. Prior to 2010, the cutoff for hormone receptor expression was 10% in many institutions, including our own. As a result, prior to 2010, biomarker studies in the literature had variable hormone receptor reporting, with negative hormone receptor tumor staining that ranged from <1% to <10%.

For HER2, the American Society of Clinical Oncology and the College of American Pathologists published guidelines in 2007 (ASCO/CAP 2007 guidelines), which included modifications to IHC analysis and fluorescence in-situ hybridization (FISH) tests that reverted back to the Food & Drug Administration (FDA) guidelines in 2013 [24,25]. Previously, our group compared concordance rates before and after the 2007 guidelines. Prior to 2007, the concordance rate between the assays was 97.6% with a corresponding kappa coefficient of 0.90. After 2007, the concordance rate was 97.6% with a corresponding kappa coefficient of 0.89. The implementation of the new ASCO/CAP 2007 HER2 guidelines did not show a significant difference in concordance rates and did not decrease the number of inconclusive cases [26].

More recently a similar study by Overcast et al. [27], demonstrated that by using the 2013 ASCO/CAP HER2 guidelines, there was a significant shift in HER2 FISH recategorization on core needle biopsy, reclassifying 9.9% of cases. Most (9.3%) changed from the HER2 FISH negative category to the HER2 FISH equivocal category, resulting in an increase of HER2 equivocal cases from 0.5% to 6.5% [27].

The ASCO/CAP 2007 guidelines for HER2 also introduced an average HER2 copy number criterion for HER2 amplification of ≥6.0/cell as positive for amplification. New guidelines increased HER2 positive cases by 2.6%, increased equivocal cases by 2.1% and decreased HER2 negative cases by 4.1%, according to Singh et al. [28]. Lim et al. [29] found similar results, showinga decrease in HER2 negative and an increase in HER2 positive as well as equivocal cases, with an overall decrease in concordance rates between FISH and IHC from 94.9% to 93.8%.

Instability of tumor biomarkers throughout tumor progression and with multiple relapses

The mechanism behind alterations in biomarkers after neoadjuvant chemotherapy is not well understood. It may be due to chemotherapy effects on selectively killing sensitive cancer cells and leaving insensitive cells behind in residual tumors, or it may be secondary to intratumoral heterogeneity. It is documented that alterations of biomarkers are more frequently seen after neoadjuvant chemotherapy than in control groups. Gahlaut et al. [30], compared neoadjuvant treated groups with control, non-neoadjuvant treated groups and found more biomarker alterations after neoadjuvant chemotherapy. Statistical significance in this study, however, was only seen with PR, and not with ER or HER2 [30]. Overall, the literature has suggested that Ki-67, followed by PR are altered more frequently than HER2 and ER. Triple negative breast cancers are known to stay as triple negative tumors after neoadjuvant chemotherapy [31]. Our data also showed statistically significant differences in Ki-67 and mitotic rates after neoadjuvant chemotherapy, as has been seen in other studies [32,33].

Pathologic Complete Response (pCR) after neoadjuvant chemotherapy occurs more frequently for patients with HER2 positive and triple negative breast cancers when compared to hormonal receptor positive tumors. pCR is also a predictive factor of better overall survival and disease free survival, especially when seen with changes in Ki-67 expression and axillary lymph node response [31].

As far as prognosis is concerned, loss of hormonal receptor positivity and switching to triple negative phenotype were related to worse overall survival and disease-free survival [8,34]. [18]The current study evaluated overall survival between groups based on pre- and post-neoadjuvant chemotherapy biomarker statuses, and showed statistically significant differences between PR groups. Alterations in biomarkers are not unique to the neoadjuvant setting. Curtit et al. [35], reported ER and PR changes in 17% and 29%, respectively, between the primary breast tumor and the corresponding metastatic lesion after adjuvant chemotherapy. Interestingly, in this paper, a positive cut off for ER and PR was 10%. Currently HER2 remained stable, and only 4% discordance was reported with HER2/*neu* in their study.

Regardless of the mechanisms underlying discordances between pre- and post-treatment biomarker statuses, most studies have recommended re-testing hormone receptors and HER2/*neu* expression on surgical specimens received after neoadjuvant chemotherapy. A loss of ER and HER2 expression and amplification could imply resistance to endocrine therapy or trastuzumab, respectively. Likewise, gain in expression of ER or HER2 may introduce additional therapeutic options, providing improved prognosis [36,37]. Accordingly, given the treatment and prognostic implications of changed biomarker status after neoadjuvant chemotherapy, we recommend repeating biomarker studies on resection specimens to most appropriately guide adjuvant chemotherapy decisions.

In summary, we present our cohort of 195 patients who had received neoadjuvant chemotherapy and were re-tested for biomarkers on residual tumor found in excision specimens, and included clinical follow-up. We also performed an extensive literature review of discordant biomarker rates after neoadjuvant chemotherapy. Our findings are consistent with other studies, and we agree in recommending repeating biomarkers after neoadjuvant chemotherapy as in recurrent/metastatic tumors.

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