

Original Article

Correlation of HER2 overexpression with gene amplification and its relation to chromosome 17 aneuploidy: a 5-year experience with invasive ductal and lobular carcinomas

Aziza Nassar¹, Andras Khor¹, Reshmitha Radhakrishnan², Anu Radhakrishnan³, Cynthia Cohen⁴

¹Division of Anatomic Pathology, Mayo Clinic, Jacksonville, Florida; ²Cornell University School of Medicine, Ithaca, New York; ³Quest Diagnostics Laboratory, Atlanta, Georgia; ⁴Emory University School of Medicine, Atlanta, Georgia

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Abstract: The *HER2* oncogene shows expression or amplification, or both, in approximately 15% to 20% of breast cancers and has been associated with poor prognosis and a response to trastuzumab therapy. *HER2* gene status determines the eligibility of breast cancer patients for trastuzumab therapy and a large fraction (41-56%) of these patients respond to targeted therapy. Several studies have related the increased expression of *HER2* to an increased copy number of chromosome 17, rather than amplification of the *HER2* gene. We compared the results of immunohistochemistry and fluorescence in situ hybridization in both invasive ductal and invasive lobular carcinomas, to determine the frequency of chromosome 17 aneuploidy associated with discordant results. In total, 390 invasive ductal carcinomas and 180 invasive lobular carcinomas diagnosed from January 2000 to December 2005 were included in the study only if results were available for immunohistochemistry (HerceptTest; DAKO, Carpinteria, California) and fluorescence in situ hybridization (PathVysion *HER2* DNA Probe Kit; Abbott Laboratories, Des Plaines, Illinois). Tumors classified as invasive ductal carcinomas were graded according to the Bloom-Richardson grading system. Correlation between the results of immunohistochemistry and fluorescence in situ hybridization was performed for all categories. Among invasive ductal carcinomas, 29% (115/390) showed chromosome 17 aneuploidy, mostly associated with grade 3/*HER2* 2+ (45%) or grade 2/*HER2* 3+ (55%) that were not amplified. Also, 34% (12/35) of invasive lobular carcinomas showed chromosome 17 aneuploidy; approximately one-third of these cases were *HER2* 2+ (33%) and *HER2* 3+ (37%) that were not amplified. Discordance between the results of immunohistochemistry and fluorescence in situ hybridization in both ductal and lobular carcinomas is largely associated with chromosome 17 aneuploidy.

Keywords: Aneuploidy, ductal carcinoma, *HER2* genes, human chromosome pair 17, human *HER2* protein, lobular carcinoma

Introduction

The human epidermal growth factor receptor 2 (*HER2*) protein is a 185-kD transmembrane growth factor receptor with tyrosinase kinase activity involved in cellular signaling, which regulates cell growth and development [1]. The *HER2* gene is amplified in 15% to 20% of breast cancers, leading to increased expression of the protein gene product [2, 3].

Several studies have reported the utility of *HER2* protein not only as a prognostic marker but also as a therapeutic target. Overexpression of *HER2* is associated with poor prognosis,

responsiveness to trastuzumab (Herceptin; Genentech, Inc., South San Francisco, California) treatment, resistance to several chemotherapeutic agents, and early cancer recurrence and death [4]. Clinical trials have shown that trastuzumab alone or in combination with chemotherapy improves survival of patients whose tumors overexpress *HER2* [4].

Diagnostic assays for *HER2* expression in breast cancer thus have become increasingly important for both their prognostic significance and their independent guidance in therapeutic decisions that determine eligibility for trastu-

zumab-targeted treatment of breast carcinoma [1]. The 2 methods in current clinical use to assess HER2 status in breast cancer are fluorescence in situ hybridization (FISH) to evaluate *HER2* gene amplification and immunohistochemistry (IHC) to detect HER2 protein overexpression [1]. FISH is considered the reference standard and is based on a quantitative measurement of the number of copies of the *HER2* gene [2]. IHC is used primarily to determine HER2 status of breast carcinomas. According to The College of American Pathologists and American Society of Clinical Oncology guidelines, an IHC score of HER2 2+ is regarded as equivocal and should be followed by testing for *HER2* gene amplification with FISH [2, 5].

Discordance between IHC and FISH results has been reported to range from 64% to 83% in tumors scored as 2+ with IHC; these cases do not show gene amplification with FISH [6-8]. Most (90%-95%) breast carcinomas that overexpress HER2 do so secondary to *HER2* gene amplification [4, 9]. HER2 overexpression in the absence of amplification is rare (3%) [10].

Polysomy 17 (especially in highly polysomic cases; defined by gene copy number) seems to cause the inconsistency in HER2 amplification, defined by the ratio of gene copy number to chromosome copy number [11]. It is more common in nonamplified tumors with overexpression of HER2 (IHC score, 3+) than in tumors with no or low HER2 expression (IHC score, 0-1+) [11]. Polysomy 17 has been reported in 41% to 86% of HER2 nonamplified tumors scored as 2+ or 3+ overexpression with IHC [11].

Invasive lobular carcinoma (ILC) comprises 0.7% to 20% of all invasive breast carcinomas and appears to have distinct biologic factors [12]. Because the incidence of ILC is less than that of infiltrating ductal carcinoma (IDC), fewer reports address the correlation of IHC and HER2 amplification status in ILC [12]. A significantly smaller percentage of lobular cancers (13%) feature HER2 amplification than of ductal cancers (48%) [12]. HER2 amplification, when present in ILC, is a significant adverse prognostic factor [12].

We sought to determine whether a relationship exists among HER2 status with IHC, gene amplification with FISH, and chromosome 17 aneuploidy (chr 17 Aneu) in IDC and ILC.

Materials and methods

Totals of 390 cases of IDC and 180 cases of ILC diagnosed from January 2000 to December 2005 were included in the study after approval by the Emory University Institutional Review Board. All IDC cases had both IHC (HercepTest, DAKO, Carpinteria, California) and FISH (PathVysion HER2 DNA Probe Kit; Abbott Laboratories, Des Plaines, Illinois) results available for assessment. However, for ILC, select cases (19.4%; 35/180) had both results available. All cases were histologically classified according to the Bloom-Richardson grading system [13]. Patients ranged in age from 26 to 89 years (mean, 60 years). Hematoxylin and eosin-stained slides from each case were reviewed to confirm the diagnosis of either IDC or ILC. Pathology reports were used to extract the HER2 and FISH results from Emory University Hospital.

Immunohistochemistry

Antigen retrieval for HER2 with IHC is performed by immersing the slides in 10 mmol/L citrate buffer in a calibrated water bath (required temperature, 95-99°C). The slides are then incubated for 40 (\pm 1) minutes at 95°C to 99°C. After the epitope retrieval solution is decanted, the sections are rinsed in the wash buffer and later soaked in the buffer for 5 to 20 minutes before staining. The slides are loaded into the Dako autostainer using the HercepTest program, as described in the manufacturer's insert. In the autostainer, the slides are rinsed, then immersion in 200 μ L peroxidase-blocking reagent for 5 minutes, followed by rinsing and then incubation with 200 μ L primary anti-HER2 protein (or negative control reagent) for 30 minutes. They are then rinsed twice and finally immersed in 200 μ L substrate-chromogen solution (diaminobenzidine) for 10 minutes. The slides are removed from the autostainer, counterstained in hematoxylin, and coverslipped in mounting medium.

FISH was used as reference standard for HER2. For FISH analysis, the slides are deparaffinized by immersing them in CitriSolv for 10 minutes, followed by dehydration in 100% ethanol at room temperature and, finally, air-dried in a slide warmer at 45°C to 50°C. The slides are pretreated by immersing them first in 0.2 N hydrochloride for 20 minutes (room tempera-

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ture) and then in purified water for 3 minutes, wash buffer for 3 minutes, pretreatment solution at 80°C for 30 minutes, purified water for 1 minute, and wash buffer for 5 minutes. The slides then undergo protease treatment by immersion in protease solution for 10 minutes at 37°C, followed by wash buffer for 5 minutes (room temperature), and finally air-dried on a slide warmer at 45°C to 50°C. The slides are subjected to denaturation by immersion in denaturing solution at 72±1°C for 5 minutes, followed by 70% ethanol for 1 minute, 85% ethanol for 1 minute, and 100% ethanol for 1 minute. They are finally air-dried on a slide warmer at 45°C to 50°C. The slides undergo hybridization by application of 10 µL of probe mixture to the target area of the slide; a 22×22 mm glass cover slip is placed over the probe to allow even spreading, and the edges of the cover slip are sealed with rubber cement. The slides are placed in a prewarmed humidified hybridization chamber and incubated at 37°C overnight (14-18 h). After the cover slips and rubber cement are removed by gently pulling the sealant with forceps, the slides are immersed in 2XSSC/0.3% NP-40 (100 mL 20XSSC [pH, 5.3] plus 847 mL purified water plus 3 mL NP-40; pH-adjusted to 7.0 to 7.5 with 1 N NaOH) at 72±1°C for 2 minutes. The slides are then air-dried in the dark in an upright position. Next, 10 µL of DAPI counterstain is applied to the target area of the slide and a 22×40 mm glass coverslip is placed over the DAPI.

IHC positivity was indicated by the presence of dark brown membranous staining for HER2. The test's scoring system was applied: a negative score meant staining of no membrane or incomplete and faint within ≤ 10% of tumor cells; 1+, incomplete faint membrane staining in more than 10% of tumor cells; 2+, more than 10% of cells showing circumferential incomplete and/or weak moderate staining or complete and circumferential intense staining within ≤ 10% of tumor cells; and 3+, intense circumferential and complete membrane staining in more than 10% of tumor cells. Two investigators (A. N. and C. C.) evaluated the IHC results, using the new CAP/ASCO guidelines [2] for HER2 assessment.

All cases with a score of 3+ and 2+ that were amplified with FISH were considered positive for HER2. For FISH amplification, the DNA probe kit used a dual-color probe for determining the number of copies of HER2 (orange) and chro-

somosome 17 centromeres (green). A minimum of 60 nuclei were scored by 2 observers using an Olympus BX 41 fluorescent microscope with a Chroma filter set (DAPI/spectrum orange/spectrum green triple bandpass). Areas scored were limited to regions of invasive disease, as compared with a companion hematoxylin and eosin-stained section. A ratio of HER2 signals to chromosome 17 centromere signals was determined. Ratios of less than 1.8 or average HER2 gene copy number of < 4 signals/nucleus were considered nonamplified; greater than 2.2 or average HER2 gene copy number > 6 signals/nucleus, amplified; and 1.8 to 2.2 or average HER2 gene copy number 4-6 signals/nucleus, equivocal.

Specimens with a chromosome 17 copy number in the range of 1.5 to 2.25 signals per cell were defined as having disomy 17. The other cases were considered to have aneusomy 17 either hypodisomy 17 (< 1.5 signals per cell), low polysomy 17 (2.26-3.75 signals per cell), or high polysomy 17 (> 3.76 signals per cell).

Results

For IDC, the following grades were assigned: grade 1, 22.3% (87/390); grade 2, 33.8% (132/390); and grade 3, 43.8% (171/390). Grades 1 and 2 IDC with negative (0 or 1+) and 2+ HER2 with IHC showed no amplification with FISH in 86% to 91% of cases (Table 1). HER2 amplification with FISH was more common in grade 3 (58/79; 73%), which expressed HER2 3+ with IHC, and less common in grade 1 (15/37; 41%), which expressed HER2 3+ with IHC. Likewise, FISH amplification was more common in grade 3 (11/41; 27%), which expressed HER2 2+ with IHC, and less common in grade 2 (8/56; 14%), which expressed HER2 2+ with IHC.

Approximately 29.5% (115/390) of IDC cases showed ch 17 Aneu. Of those amplified (n=127), 6 (5%) had chr 17 Aneu, and of those not amplified (n=225), 109 (48%) had ch 17 Aneu. This aneuploidy was seen more commonly in grade 2 (25/48; 52%), which expressed 2+ with IHC, and similarly in grade 3 (13/30; 43%), which also expressed 2+ with IHC (Table 1). Grade 3/HER2 3+ cases of IDC were associated with concordant results with FISH in 73% of cases; whereas 29% of nonamplified cases showed chr 17 Aneu.

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Table 1. Results of HER2 IHC and FISH with chromosome 17 aneuploidy in invasive ductal carcinomas with relation to tumor grade

Tumor Grade (n=390)	HER2 IHC		FISH		Chr 17 Aneuploidy
	Score	No (%)	No. (%)		No. (%)
1 (n=87)	Negative	18 (21)	2 (11) (amp)		1 (50)
			16 (89) (nonamp)		7 (44)
	2+	32 (37)	3 (9) (amp)		2 (66)
			29 (91) (nonamp)		13 (45)
	3+	37 (42)	15 (41) (amp)		1 (7)
			22 (59) (nonamp)		11 (50)
2 (n=132)	Negative	23 (17)	2 (9) (amp)		0 (0)
			21 (91) (nonamp)		7 (33)
	2+	56 (42)	8 (14) (amp)		0 (0)
			48 (86) (nonamp)		25 (52)
	3+	53 (40)	33 (62) (amp)		1 (3)
			20 (38) (nonamp)		11 (55)
3 (n=171)	Negative	51 (30)	3 (6) (amp)		0 (0)
			48 (94) (nonamp)		16 (33)
	2+	41 (24)	11 (27) (amp)		0 (0)
			30 (73) (nonamp)		13 (43)
	3+	79 (46)	58 (73) (amp)		1 (2)
			21 (27) (nonamp)		6 (29)

Abbreviations: Amp, amplified; chr 17, chromosome 17; FISH, fluorescence in situ hybridization; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry; nonamp, nonamplified.

Table 2. Results of HER2 IHC in invasive lobular carcinomas

Results [n=180] (%)	HER2 IHC Scores (%)			
	Negative	1+	2+ ^a	3+ ^b
ER+, - 173 (96%)	98 (57%)	17 (9%)	36 (21%)	22 (13%)
ER-, - 7 (4%)	5 (71%)	0 (0%)	0 (0%)	2 (29%)

Abbreviations: ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry. ^aMost (27/36 [75%]) of these cases were reflexed to fluorescence in situ hybridization; ^bFew (4/24 [17%]) of these cases were reflexed to fluorescence in situ hybridization.

Grade 2/HER2 3+ and Grade 3/HER2 2+ cases that showed no amplification with FISH were associated with chr 17 Aneu in 55% and 43% of cases, respectively. Most IDC cases that had a HER2 IHC score of 2+ and were nonamplified appeared to be secondary to chr 17 Aneu (45% [13/29] in grade 1; 52% [25/48] in grade 2, and 43% [13/30] in grade 3). Most of the IDC cases that had a HER2 IHC score of 3+ and were nonamplified appeared to be secondary to chr 17 Aneu (50% [11/22] in grade 1, 55% [11/20] in grade 2, and 29% [6/21] in grade 3) (Table 1).

Most (96% [173/180]) of ILC cases were estrogen receptor (ER) positive; only 13% (22/173) were HER2 3+ positive with IHC (Table 2). ILC was routinely reflexed to FISH for cases that were HER2 2+ with IHC; however, all cases that were HER2 3+ with IHC were not sent for FISH analysis. For ILC, none of the ER-negative breast cancer cases were analyzed with FISH since most of them were either HER2 negative (71%) or HER2 3+ (29%) (Table 2). Only 34% (12/35) of ILC cases that were reflexed to FISH showed chr 17 Aneu (Table 3).

With ILC, of those cases that were reflexed to FISH, HER2 amplification was seen in only 1 of 4 (25%) HER2 3+ cases (Table 3). No HER2 FISH amplification was seen in cases with HER2 negative, 1+, or 2+ with IHC. Approximately one-third of the ILC cases that were HER2 2+ (37%) or 3+ (33%) with IHC and nonamplified were associated with chr 17 Aneu.

Discussion

Our results have shown that chr 17 Aneu was seen in 29.5% (115/390) of IDC cases and 34.3% (12/35) of ILC cases. Furthermore, we have noted that the highest percentage of chr 17 Aneu in IDC was seen in patients with an IHC score of 2+ (45%-52%) and 3+ (29%-55%). Similarly, in ILC, the highest percentage of chr 17 Aneu was seen in patients with an IHC score of 2+ (37% [10/27]) and 3+ (33% [1/3]). Thus, chr 17 Aneu may have an important role in IDC and ILC with discordance between IHC and FISH results regarding HER2 status.

HER2 overexpression predicts a response to certain chemotherapeutic agents, such as the anthracyclines and paclitaxel. Also, such overexpression is considered a strong predictive marker for clinical benefit from HER2-targeted therapy (trastuzumab) in metastasis and, more

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Table 3. Results of FISH and chromosome 17 aneuploidy in invasive lobular carcinomas

Results [n=35]	FISH [n=35] (%)	Chr 17 Aneuploidy [n=12] (%)
ER+ and HER2 negative [n=2]		
Amp	0 (0%)	0 (0%)
Nonamp	2 (100%)	0 (0%)
ER+ and HER2 1+ [n=2]		
Amp	0 (0%)	0 (0%)
Nonamp	2 (100%)	1 (50%)
ER+ and HER2 2+ [n=27]		
Amp	0 (n=0)	0 (0%)
Nonamp	27 (100%)	10 (37%)
ER+ and HER 3+ [n=4]		
Amp	1 (25%)	0 (0%)
Nonamp	3 (75%)	1 (33%)

Abbreviations: Amp, amplified; chr 17, chromosome 17; ER, estrogen receptor; FISH, fluorescence in situ hybridization; HER2, human epidermal growth factor receptor 2; nonamp, nonamplified.

recently, in the adjuvant clinical setting [4]. HER2 positivity correlates significantly with increasing grade of IDC, p53-positive tumors, and negative ER and progesterone receptor status [14]. HER2 amplification is seen more in lymph node-positive (52%) than lymph node-negative (22%) disease, and the difference is statistically significant ($P=0.0001$) [12].

The concordance rate between FISH and HER2 2+ ranges from 17% to 72% and between FISH and HER2 3+ from 51% to 100% [7, 8, 15-18]. False-positive HercepTest results have been reported in 12% to 23% of cases [7, 19]. The HercepTest has been shown to give false-negative results in up to 28% of HER2 FISH-positive cases [19]. Reportedly, FISH for HER2 has false-negative results in 3% to 12%, possibly because of single-gene overexpression, which is reported in 2% to 13% of cases with HER2 3+ IHC staining [20].

Numerical aberrations of chromosome 17 are found in 65% of HER2-amplified tumors (single centromeric signal, 44%; > 3 centromeric signals, 21%) and 60% of unamplified cancers (single centromeric signal, 43%; > 3 centromeric signals, 17%) [21]. The trastuzumab response rate was 92% for patients with more than 2 centromeric signals (chromosome 17 eusomy or polysomy) and 53% for patients whose tumor showed a single signal (chromosome 17 monosomy) ($P=0.005$) [21]. In a study of 1,556 breast tumors, Perez et al. [22] found that 25%

(54/216) of tumor specimens scored as HER2 2+, had extra copies of *HER2* gene (aneusomy 17).

Polysomy 17 is a major cause of equivocal HER2 testing results with FISH [23], which has been reported in 8% to 39% of breast cancer cases [2, 7, 24]. HER2 expression that is equivocal to low level in breast cancer is associated, in most cases, with chromosome 17 polysomy and a corresponding increase in the *HER2* gene copy numbers (increase, 4-6) [2, 24].

The correlation of HER2 protein overexpression is highest with *HER2* gene copy number and lowest with chromosome 17 copy number [25]. In an analysis of 189 breast specimens with use of PathVysion FISH assay, 51.3% (97/189) of cases showed

aneusomy 17 (≥ 2.25 signals per cell); 43.5% (82/189), relatively low polysomy 17 (2.26-3.75 signals per cell); 5.3% (10/189), high polysomy 17 (≥ 3.76 signals per cell); and 2.6% (5/189), hypodisomy (≤ 1.75 signals per cell) [25].

High polysomy (≥ 4 chromosome 17 signals per nucleus) seems to be more strongly associated with HER2 overexpression (IHC score, 3+) and chromosome 1 copy number than low polysomy (< 4 chromosome 17 signals per nucleus) [11]. Polysomy 17 has been reported in 23% to 86% of HER2 nonamplified tumors that had an IHC score of 2+ or 3+ [26, 27].

Chromosome 17 polysomy is more common in invasive than noninvasive and preinvasive breast lesions [11]. Polysomy of chromosome 17 is also common in cases with equivocal HER2 protein expression and *HER2* gene amplification, as well as cases with discrepant HER2 protein and *HER2* gene copy number measurements [11]. The overall frequency of borderline or low-level HER2 amplification (ratio, 1.7-2.5) is observed in 8.5% to 16% of sporadic tumors with equivocal results (HER2 2+ with IHC) [24]. Borderline or low-level HER2 amplification is more often seen in BRCA1-associated breast cancers than in sporadic breast cancers [28]. The increased frequency of polysomy 17 is observed in cases with IHC scores of 3+ but not in cases with IHC scores of 0 to 2+ [28].

Aneusomy 17 is observed mainly in the form of low polysomy 17 (34.9%) but occasionally in high polysomy 17 (7.3%) [29]. Ma et al. [29] found a greater incidence of polysomy 17, particularly high polysomy 17, in cases with an IHC score of 3+ and nonamplified than in cases with an IHC score of negative, 1+, or 2+ and nonamplified [29]. They detected 51.2% (457/893) aneusomy when analyzing chromosome 17 copy number, 8.9% (80/893) hypodisomy, 34.9% (312/893) low polysomy, and 7.3% (65/893) high polysomy [29].

Tumors that show polysomy 17 in the absence of *HER2* gene amplification resemble more closely *HER2*-negative tumors than *HER2*-positive tumors [23]. Chibon et al. [26] found no significant correlation between chromosome 17 polysomic cases and such other pathologic variables as tumor grade, mitotic count, tumor differentiation, nuclear atypia, and ER and progesterone receptor status [26]. However, Krishnamurti et al. [27] found that invasive breast carcinomas with unamplified chromosome 17 polysomy are associated with several adverse prognostic indicators, such as higher nuclear grade, mitotic activity, Nottingham score, histologic grade, tumor stage, and ER negativity with a trend toward the amplified group, in contrast to patients with neither amplification nor polysomy. In general, high tumor grade is associated with polysomy 17 [11].

Monosomy 17 is a predictor of cancer aggressiveness [11]. It also has been associated with nodal metastasis [11]. Studies have shown that both monosomy and polysomy are associated with ER negativity [11]. Chromosome 17 monosomy is more common in noninvasive and pre-invasive cancers than in invasive breast lesions [11]. Chromosome 17 monosomy is independently associated with reduced response rate compared with chromosome 17 eusomy or polysomy (odds ratio, 0.091; 95% confidence interval, 0.014-0.581) [21].

Overexpression of *HER2* with IHC without gene amplification may be due to high polysomy of chromosome 17 or may occur through various molecular epigenetic or posttranscriptional events (e.g., DNA hypomethylation in the promoter regions, posttranscriptional events) [18]. In conclusion, for a significant number of IDC and ILC cases specifically, those with IHC

scores of 2+ and 3+ with discordance between IHC and FISH results, the discordance is due to chr 17 Aneu.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Aziza Nassar, Division of Anatomic Pathology, Mayo Clinic, 4500 San Pablo Road, Mayo 3-160S, Jacksonville, Florida 32224, America. E-mail: nassar.aziza@mayo.edu

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