RESEARCH ARTICLE

Assessment of *HER2/neu* level using molecular cytogenetic in breast cancer: A study at a regional cancer center

Dharmesh M. Patel, Ranjan A. Parmar, Priya K. Varma, Dhara C. Ladani, Prabhudas S. Patel, Pina J. Trivedi

Patel DM, Parmar RA, Varma PK, et al. Assessment of HER2/neu level using molecular cytogenetic in breast cancer: A study at a Regional Cancer Center. J Genet Mutat 2018;1(2):21-24.

ABSTRACT

Background: Amplification of *HER2/neu* gene observed in 25–30% of breast cancer patients. FDA approved Trastuzumab is only effective drug used in tumor having the *HER2/neu* amplification. Fluorescence *In Situ* Hybridization (FISH) and immunohistochemistry (IHC) are established gold standard techniques widely used to study *HER2/neu* gene status and their protein expression. Cases with equivocal 2+ using IHC technique showed discordant results with FISH technique. Present study was organized to establish the frequency of *HER2/neu* gene status by FISH in score 2+ by IHC and to compare these results with clinicopathological parameters in breast carcinoma patients.

Methods: Fifty breast cancer patients scored as 2+ by IHC were included in the study. ER and PR status were evaluated for all the enrolled cases. FISH was performed using ZytoLight SPEC HER2/CEN 17 Dual Color Probe Kit. ASCO/CAP (American Society of Clinical Oncology /College of American Pathologists) guidelines were used for analysis. Statistical Analysis (Spearman Correlation, Chi-Square) was done by SPSS software version 23.

Results: Out of fifty patients, twenty-eight (56%) were positive for gene amplification while 19 (38%) were negative (ratio ranging from 1 to 10), gene amplification correlated with age, histological grade, and lymph node status. Considerable inverse association obtain between HER2/neu gene amplification and ER (P=0.03, r=-0.3), PR status (P 0.05, r=-0.2).

Conclusion: *HER2/neu* amplification status can reliably determine by FISH especially in equivocal cases by IHC. Accurate assessment of gene with location and their copies in individual cell reveled by FISH. The overall concordance rate between both FISH and IHC results underline the very fact that FISH is gold standard for precise assessment of HER2/neu gene amplification in IHC-equivocal breast carcinomas.

Key Words: *HER2; Breast; FISH; IHC; Cancer; Equivocal.*

Abbreviations: *HER2*: Human Epidermal Group Factor Receptor 2; EGFR: Human Epidermal Growth Factor Receptor; IHC: ImmunoHistoChemistry; FISH: Fluorescence *in-situ* hybridization

Throughout the world, breast carcinoma is the most commonly diagnosed cancer in women and leading reason of cancer-related deaths in women [1]. *HER2/neu* is the member of the Human Epidermal Growth Factor Receptor (*EGFR*) family encoded by a gene located on the long arm of chromosome 17 (17q12–21.32) [2]. On the basis of specific markers, totally different histologic subtypes can be characterized. Estrogen Receptor (ER), Progesterone Receptor (PR), and Human Epidermal Group Factor Receptor 2 (*HER2*) are most ordinarily studied known markers [3,4]. Patients with the *HER2/neu* positive disease typically represent cancer type with special biological behavior and clinical features with aggressive tumor progress and poor survival. Existing therapies such as trastuzumab (Herceptin®) and Lapatinib (Tyverb/Tykerb®), a monoclonal antibody inhibitor and a dual *EGFR/HER2* kinase inhibitor, respectively, are currently used in the treatment of *HER2/neu* positive cancers [5]. However, *HER2/neu* tumor diversity could be a huge challenge for proper assessment of the *HER2/neu* gene sequence which may affect the treatment of early and advanced breast cancer patients [6]. Recent studies have reported that *HER2/neu* gene amplified patients had decrease disease-free survival [7]. It was more often observed in IHC 2+/equivocal cases. It was also observed negative for *HER2/neu* gene amplification cases, containing hardly one or two individual amplified tumor cells [8].

Precise estimation of *HER2/neu* level in individual tumor cells is required before application of specific therapeutic agent and therefore *HER2/neu* amplification test has great importance in breast cancer. Currently two techniques widely used for detection of *HER2/neu* gene. IHC used for protein over expression while FISH used for gene amplification. FISH allows rapid image within the cell morphology and even in single cell assessment and distribution of gene in the histological section. FISH has tremendous sensitivity and specificity both in detecting *HER2/neu* amplification however with extremely essential of special instrumentation and experience to perform and interpret the results [9].

The aim of present study was to assess the frequency of *HER2/neu* gene amplification in IHC equivocal breast cancer patients using FISH technique and to compare these findings with Estrogen Receptor (ER), Progesterone Receptor (PR) status and clinicopathological parameters..

METHODOLOGY

Study design

The present study included 50 IHC 2+/equivocal cases of invasive breast cancer registered at the Gujarat Cancer and Research Institute, a regional cancer and research institute, Gujarat, India. Formalin fixed tissue were used for study. Only patient with sufficient tumor cells were included in the study. 2-4 μm thick tissue sections were cut from a paraffin block and applied to positively charged slides. *HER-2* protein expression was measured using a commercially available reagent on Ventana Benchmark XT auto stainer (Ventana, USA). Commercial available double-color FISH probe was used for *HER-2/neu* gene amplification assessment.

IHC analysis

IHC study was done on paraffin embedded, formalin-fixed tissue sections using Ventana Benchmark XT autostainer using Ventana reagents (Ventana, USA), using the manufacturer's protocol. ASCO/CAP guidelines were used for analysis [10]. Cells with membranous positivity were considered as *HER-2* protein over expression. Scoring was done by pathologists according to the manufacturer's recommendations, in order to truly reflect the concordance or discordances between IHC and FISH.

FISH for *HER-2/neu* gene amplification

FISH was performed using ZytoLight SPEC *HER2/CEN* 17 Dual Color Probe Kit (ZytoVision GmbH, Fischkai1, D-27572 Bremerhaven, Germany). It was consisted of two *DNA* probes: The probe contained green-labeled

Cytogenetic Lab, Department of Cancer Biology, Gujarat Cancer & Research Institute, Asarwa, Ahmedabad, Gujarath, India

Correspondence: Trivedi PJ, Cytogenetic Lab, Department of Cancer Biology, Gujarat Cancer & Research Institute, Asarwa, Ahmedabad, Gujarath, India, Telephone +91-79-22688364, e-mail pjt1410@rediffmail.com

Received: September 27, 2018, Accepted: October 16, 2018, Published: October 23, 2018

polynucleotides for *HER2* gene and orange-labeled polynucleotides for the centromere of chromosome 17. FISH assay was performed as per the manufacturer's instructions.

FISH analysis was done by two molecular cytogenetic experts without prior knowing the clinical diagnosis. The slides scanning and capturing were done by using OLYMPUS BX 51 fluorescent microscope (OLYMPUS BX51, Japan). Total Twenty randomly selected invasive tumor cells were evaluated for reporting. Cells were scored as per mentioned in Table 1.

In case of equivocal result, more randomly selected tumor nuclei were evaluated by other evaluator.

RESULTS

Total 50 breast cancer patients were enrolled in the present study; out of that 90% patients were diagnosed as invasive ductal carcinoma. Patients were in the age range of 28-85 years with a mean age of 52 years. 82% patients were in the postmenopausal status while 18% were in premenopausal status, predominantly in 54% patients Grade II tumors were seen; and grade III were seen in 18% patients. Cases with Stage II and Stage III were more frequent (44% and 38% respectively), while cases with stage I and IV were less common (2% and 6% respectively). "ER+ PR+" (ER positive and PR positive) status was seen in 48% of the patients, while "ER- PR-" (ER negative and PR negative) status was seen in 38% patients. Lymph nodes were involved in 56% patients (Table 2)..

HER2/neu gene level was correlated with different clinico-pathological parameters such as grade, stage of the disease, age, menopausal status, tumor size, nodal status, and hormone receptor status.

In the present study *HER2/neu* amplification was increased with grade and tumor stage. Gene amplification also correlated with lymph node positivity as compared to lymph node negativity, 28 cases was positive for nodal involvement, from those 68% patients showed gene amplification. 14 (73.6%) patients out of 19 ER negative patients showed gene amplification by FISH which was inversely correlated, also statistically significant (P=0.03, r=-0.3). But there was no significant result found between ER positivity and gene amplification as the number of patients showing amplification and non-amplification are similar. ER, PR also had inverse correlation, from 26 negative cases 18 (69.2%) showed gene amplification which was also statistically significant (P=0.05, r=-0.2), while 7 (26.9%) were negative for amplification (Table 3).

Out of 50 equivocal IHC cases, 28 (56%) patients were positive for gene amplification while 19 (38%) were negative, 2 cases showed equivocal result by FISH. Assay was repeated for both cases. Results were positive for gene amplification in both cases. 1 case was failed for FISH assay may be due to inappropriate tissue processing or prolonged storage of paraffin block (Table 3).

From 50 cases, there were only 4(8%) ER/PR negative cases and also negative for gene amplification. 14 (73.6%) cases were ER/PR negative but

TABLE 1

FISH *HER2/neu* scoring as per ASCO/CAP guidelines

Interpretation	*HER-2/neu* to CEP 17 ratio
Negative	ratio <2 and average *HER-2* copy numbers less than 4 signals/cell
Equivocal	ratio <2 and average *HER-2* copy numbers ≥ 4 to <6 signals/cell
Positive	ratio ≥ 2 or ratio <2 and average *HER-2* copy numbers ≥ 6 signals/cell

TABLE 2

Clinicopathological characteristics of total 50 breast Cancer patients

Variables	Data number (%)
Age range	28-85 years
Age <45	16 (32)
Age >45	34 (68)
Menopausal status	
Premenopausal	09 (18)
Postmenopausal	41 (82)
Histology	
Grade I	04 (08)
Grade II	27 (54)
Grade III	09 (18)
Non informative	10 (20)
Stage of disease	
Stage I	01 (02)
Stage II	22 (44)
Stage III	19 (38)
Stage IV	03 (06)
Non informative	05 (10)
Lymph node involvement	
Node positive	28 (56)
Node negative	16 (32)
Non informative	06 (12)
Hormone receptor	
ER+ PR+	24 (48)
ER- PR-	19 (38)
ER+ PR-	07 (14)
ER- PR+	00 (00)

TABLE 3

Correlation of HER2 status with clinicopathological characteristics

Variables	*HER2* - number (%)					
	Amplified	Non amplified	Equivocal	Non Informative	P value	r value
Grade						
I(n=4)	02 (50)	02 (50)	-	-		
II (n=27)	15 (57.7)	11 (42.3)	-	-	0.25	0.18
III (n=9)	07 (77.7)	02 (22.2)	-	-		
Non informative (n=10)	06 (60)	04 (40)	-	-		
Stage						
I(n=1)	-	01 (100)	-	-		
II (n=22)	10 (45.4)	11 (50)	01 (4.5)	-	0.08	0.26
III (n=19)	13 (68.4)	05 (26.3)	-	01 (5.2)		
IV (n=3)	02 (66.6)	01 (33.3)	-	-		
Non informative (n=5)	03 (60)	01 (20)	01 (20)	-		
Lymph node						
Positive (n=28)	19 (67.8)	08 (28.5)	01 (3.5)	-	0.27	0.19
Negative (n=16)	06 (37.5)	09 (56.2)	01 (6.2)	-		
Non informative (n=6)	03 (50)	02 (33.3)	01 (16.6)	-		
Age						
>45 (n=34)	21 (61.7)	12 (35.2)	01 (2.9)	-	0.33	0.14
<45 (n=16)	7	7	01 (6.2)	01 (6.2)		
Menopausal status						
Premenopausal (n=09)	04 (44.4)	03 (33.3)	01 (11.1)	01 (11.1)	0.7	0.04
Postmenopausal (n=41)	24 (58.5)	16 (39)	01 (2.4)	-		
Hormone status						
ER						
Positive (n=31)	14 (45.1)	15 (48.3)	01 (3.2)	01 (3.2)	0.03	-0.3
Negative (n=19)	14 (73.6)	04 (21)	01 (5.2)	-		
PR						
Positive (n=24)	10 (41.6)	12 (50)	01 (4.1)	01 (4.1)	0.05	-0.27
Negative (n=26)	18 (69.2)	07 (26.9)	01 (3.8)	-		

showed gene amplification. There was no notable difference between ER/PR positive cases and gene amplification (Table 4).

TABLE 4

Comparison of HER2 gene amplification with combined ER/PR status

ER/PR Status by IHC	*HER2* amplification by FISH number (%)			
	Amplified	Non amplified	Equivocal	Non Informative
ER+ PR+ (n=24)	10 (41.6)	12 (50)	01 (4.1)	01 (4.1)
ER- PR- (n=19)	14 (73.6)	04 (21)	1 (5.2)	-
ER+ PR- (n=07)	04 (57.1)	03 (42.8)	-	-
ER- PR+ (n=00)	-	-	-	-

DISCUSSION

US Food and Drug Administration were accepted that *HER2/neu* gene expression is a significant predictor for response to some therapy. Trastuzumab (Herceptin), a humanized monoclonal anti–*HER2* antibody and chemo adjuvant therapy used for patients with metastatic breast cancer over expressing *HER2/neu* protein [11]. To estimate of *HER2/neu* gene status become most important part of pathologic report of breast cancer. Although some evidence that FISH testing predicts the therapeutically significant *HER2* status more correctly, the approach of primary IHC screening with additional FISH molecular verification is broadly used [12]. No statistically significant results obtained between *HER2/neu* gene amplification and age, grade or lymph nodes metastasis in present study. This was also mentioned by other study group [12].

Gene amplification showed positive correlation with high grade tumors as compared to low grade tumors. Panjwani et al. showed positive correlation between grade III and *HER2* amplification which is concordant with existing literature [11].

In the present study patients with advanced stage (stage III) showed frequent gene amplification than patient in early stage (stage I and II). Zubair Ahmed et al. documented gene amplification in 4.17% patients of grade 1 tumors, 75.83% patients of grade 2 and 20% patients of grade 3 tumors [13]. Mudduwa reported gene amplification in 14.6% patients of grade 1 tumors, 36.4% patients of grade 2 tumors and 49% patients of grade 3 tumors [14]. The grade 1, grade 2 and grade 3 tumors in the study by Lobna Ayadi et al. was 10.9%, 63.2% and 25.8% [15].

Present study showed contrary association between hormone receptor and *HER-2* gene amplification (Table 3) possibly may be due to the complex signaling between ER and other growth factor signaling pathways in breast cancer cells. In present study, ER positive and *HER2/neu* amplification was observed in 45% cases. Gunn et al. have reported *HER2/neu* amplification with ER positive cases with resistance to tamoxifen therapy [16]. It is believed that, tamoxifen functions as an estrogen agonist to improve growth in breast cancer cells which express high levels of *HER2/neu* and estrogen receptor co-activator resulting in resistance for Tamoxifen [17].

In the present study we have reported 56% cases with *HER-2/neu* amplification among IHC 2+ patients. In accordance to our finding, another group in India reported 66.6% cases of *HER-2/neu* amplification in IHC 2+ cases in their study [18].

In our study findings of *HER-2/neu* gene amplification (56%) by FISH and protein expression by IHC indicates very weak association particularly in IHC equivocal cases. This highlights the fact that FISH is the standard technique for *HER-2/neu* gene amplification study particulary in IHC-equivocal breast cancer patients [19]. However, larger number of cases needs to be study before stating any conclusion. In current study triple negatives i.e., negative for ER, PR and *HER-2* gene, were reported in 8% patients. Triple negative patients are resistant to chemotherapy and indication of more aggressive tumor also need more advance clinical course to overcome less effective conventional chemotherapy [20-22]. In the current study 4% cases shows equivocal FISH results. This is in correlation with the 2013 ASCO/CAP guidelines [23]. Patients in this category also need to be treated from trastuzumab therapy [24].

The debate on the best method to conclude *HER-2/neu* status, we find that the *HER-2/neu* status can be reliably determined by FISH method and allows the precise cell by cell evaluation in these samples [25]. Investigation of *HER-2/neu* amplification would be helpful in determining prognostic factors. The equivocal cases investigated by both methods should be monitored on follow up study. The present study strongly recommended combined approach using both techniques can be a optimize test. In conclusion, the present study highlights the significance and remarkable nature of FISH technique as gold standard for determination of *HER2/neu* status in breast cancer..

CONFLICT OF INTEREST

There are no conflicts of interest to disclose from all authors.

REFERENCES

1. Kurozumi S, Padilla M, Kurosumi M, et al. HER2 intratumoral heterogeneity analyses by concurrent HER2 gene and protein assessment for the prognosis of HER2 negative invasive breast cancer patients. Breast Cancer Res Treat. 2016;158:99-111.
2. Ferlay J, Shin HR, Bray F, et al. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer. 2010;127:2893-917.
3. Bertos NR, Park M Breast cancer-one term, many entities? J Clin Invest. 2011;121:3789-96.
4. Citri A, Yarden Y. EGF–ERBB signalling: Towards the systems level. Nat Rev Mol Cell Biol. 2006;7:505-16.
5. Schroeder RL, Stevens CL, Sridhar J. Small molecule tyrosine kinase inhibitors of ErbB2/HER2/Neu in the treatment of aggressive breast cancer. Molecules. 2014;19:15196-212.
6. Hanna WM, Rüschoff J, Bilous M, et al. HER2 *in situ* hybridization in breast cancer: Clinical implications of polysomy 17 and genetic heterogeneity. Mod Pathol. 2014;27:4-18.
7. Seol H, Lee HJ, Choi Y, et al. Intratumoral heterogeneity of HER2 gene amplification in breast cancer: Its clinicopathological significance. Mod Pathol. 2012;25:938-48.
8. Lee HJ, Seo AN, Kim EJ, et al. HER2 heterogeneity affects trastuzumab responses and survival in patients with HER2-positive metastatic breast cancer. Am J Clin Pathol. 2014;142:755-66.
9. Saez A, Andreu FJ, Segui MA, et al. HER-2 gene amplification by chromogenic *in situ* hybridisation (CISH) compared with fluorescence *in situ* hybridisation (FISH) in breast cancer-A study of two hundred cases. The breast. 2006;15:519-27.
10. Wolff AC, Hammond ME, Schwartz JN, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. Arch Pathol Lab Med. 2007;131:18-43.
11. Goud KI, Dayakar S, Vijayalaxmi K, et al. Evaluation of HER-2/neu status in breast cancer specimens using immunohistochemistry (IHC) & fluorescence *in-situ* hybridization (FISH) assay. Indian J Med Res. 2012;135:312-7.
12. Mostafa NA, Eissa SS, Belal DM, et al. Assessment of *Her*-2/neu gene amplification status in breast carcinoma with equivocal 2+ *Her*-2/neu immunostaining. J Egypt Natl Canc Inst. 2011;23:41-6.
13. Ahmad Z, Khurshid A, Qureshi A, et al. Breast carcinoma grading, estimation of tumor size, axillary lymph node status, staging, and nottingham prognostic index scoring on mastectomy specimens. Indian J Pathol Microbiol. 2009;52:477-81.
14. Mudduwa LK. Quick score of hormone receptor status of breast carcinoma: Correlation with other clinicopathological prognostic parameters. Indian J Pathol Microbiol. 2009;52: 159-63.
15. Ayadi L, Khabir A, Amouri H, et al. Correlation of *HER-2* over-expression with clinico-pathological parameters in Tunisian breast carcinoma. World J Surg Oncol. 2008;6:112.
16. Gunn S, Yeh IT, Lytvak I, et al. Clinical array-based karyotyping of breast cancer with equivocal HER2 status resolves gene copy number and reveals chromosome 17 complexity. BMC cancer. 2010;10:396.
17. Panjwani P, Epari S, Karpate A, et al. Assessment of HER2 status in breast cancer using Fluorescence *in situ* hybridization and immuno-chemistry: Experience of a tertiary cancer referral centre in India. Indian J Med Res. 2010;132:287-94.
18. Tubbs RR, Pettay JD, Roche PC, et al. Discrepancies in clinical laboratory testing of eligibility for trastuzumab therapy: apparent immunohistochemical false-positives do not get the message. J Clin Oncol. 2001;19:2714-21.
19. Rakha EA, Reis-Filho JS, Ellis IO Basal-like breast cancer: A critical review. J Clin Oncol. 2008;26:2568-81.
20. Wolff AC, Hammond ME, Schwartz JN, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 resting in breast cancer. J Clin Oncol. 2007;25:118-45.
21. Long TH, Lawce H, Durum C, et al. The new equivocal: Changes to HER2 FISH results when applying the 2013 ASCO/CAP guidelines. Am J Clin Pathol. 2015;144:253-62.
22. Wolff AC, Hammond ME, Schwartz JN, et al. American Society of Clinical Oncology/College of American pathologists. Guideline recommendations for human epidermal growth factor receptor testing in breast cancer. Arch Pathol Lab Med. 2007;131:18-43
23. Salido M, Tusquets I, Corominas JM, et al. Polysomy of chromosome 17 in breast cancer tumors showing an overexpression of ERBB2: A study of 175 cases using fluorescence *in situ* hybridization and immunohistochemistry. Breast Cancer Res. 2005;7:67-73.
24. Watters AD, Going JJ, Cooke TG, et al. Chromosome 17 aneusomy is associated with poor prognostic factors in invasive breast carcinoma. Breast Cancer Res Treat. 2003;77:109-14.
25. Hoang MP, Sahin AA, Ordonez NG, et al. HER-2/neu gene amplification compared with HER-2/neu protein overexpression and interobserver reproducibility in invasive breast carcinoma. Am J Clin Pathol. 2000;113:852-9.