

# Clinical role of *HER2* gene amplification and chromosome 17: a study on 154 IHC-equivocal cases of invasive breast carcinoma patients

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Received: 9 May 2014 / Accepted: 25 August 2014 / Published online: 6 January 2016  
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**Abstract** Accurate evaluation of human epidermal growth factor receptor 2 (*HER2*) status is quite crucial for invasive breast tumor patients in order to select anti-*HER2* therapy for effective clinical outcomes. Immunohistochemistry (IHC) assay is routinely used to evaluate the *HER2* oncoprotein overexpression but is unable to explain the chromosomal and genetic alterations and has been considered as a hot issue in IHC-equivocal cases. We investigated these molecular aberrations in correlation with prognostic factors. A cohort of 154 IHC-equivocal (+2) cases was selected and retrospectively analyzed by dual-probe fluorescence in situ hybridization (FISH) assay by using locus-specific *HER2* and centromere enumeration probes (CEP17) for the identification of *HER2* proto-oncogene amplification and chromosomal copy number per cell, respectively. The data were analyzed by SPSS 16.0 version using chi-square test ( $p < 0.05$ ). We identified 36 out of 154 cases (23.4 %) showing *HER2* gene amplification (average *HER2* gene copies per cell  $>4$  or  $<4$  with *HER2*/CEP17 ratio  $>2$ ) in concordance with *HER2* oncoprotein overexpression, and significant correlation was observed with prognostic parameters including histological type, tumor

grade II to III, histology and pathological type, lymphatic invasion, ductal carcinoma in situ (DCIS), and estrogen-positive and progesterone-negative receptors. Of the 154 cases, 18 cases (11.7 %) showed polysomy 17 with CEP17 probe signals per cell  $\geq 3$  and 22 cases (14.3 %) presented monosomy 17 (CEP17 probe signals per cell  $\leq 1$ ). Our data indicate that the use of anti-*HER2* therapy should not be suggested unless true evaluation of *HER2* protein expression is made regarding gene amplification essentially in IHC-ambiguous invasive breast tumors.

**Keywords** *HER2* · Breast cancer · Prognosis · Dual-probe fluorescent in situ hybridization · FISH

## Introduction

In Pakistan, among the top ten commonest malignancies, breast cancer is the leading one and ranks second in position worldwide [1]. Invasive breast cancer constitutes 75–80 % of breast tumors [2] and 15–30 % is being caused by *HER2* gene amplification/overexpression [3, 4]. Human epidermal growth factor receptor 2 (*HER2*) gene is located at chromosome 17 q12–21.32 and acts as a proto-oncogene involved in modulating proliferation, invasion, and apoptosis [5]. It encodes a p185 *HER2* oncoprotein, also known as *HER-2/neu* or *erbB-2*, an important member of the *HER* family which is a cell membrane receptor having tyrosine kinase activity that can stimulate cell proliferation through the RAS–MAPK downstream signaling cascades and inhibit cell death through the phosphatidylinositol 3'-kinase–AKT–mammalian target of rapamycin (mTOR) pathway [6].

The overexpression of *HER2* protein may result by increased gene copy numbers (amplification) under different

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aberrative genetic mechanisms such as increased replicating amplicons (repeated *HER2* gene segments) on chromosome 17 and chromosomal aneusomy which could associate with various types of cancers remarkably breast, ovarian, gastric, pancreatic, prostate, colorectal, and lung cancer and tumors of the female genital tract [3, 7].

Invasive breast cancer patients with *HER2* protein overexpression are being subjected to trastuzumab therapy (Herceptin, Genentech), a recombinant monoclonal antibody to target the *HER2* extracellular domain to suppress the downstream signaling pathways [8]. Trastuzumab is a Food and Drug Administration (FDA)-approved drug and has been recommended as a monotherapy or in combination with adotrastuzumabemtansine [9] for first-line treatment of *HER2*-positive invasive breast tumors [10, 11].

American Society of Clinical Oncology and College of American Pathologists (ASCO/CAP) has recommended new guidelines for diagnostic approach of *HER2* protein overexpression by immunohistochemistry (IHC) categorized with IHC score as 0/1+ (IHC-negative), 2+ (IHC-equivocal), and 3+ (IHC-positive). The IHC-equivocal or borderline 2+ cases have ambiguous tumor nature with weak *HER2* oncoprotein expression in this subgroup and highly crucial for medical practitioners to mark the patient's eligible for Herceptin therapy. In situ hybridization techniques are standard alternative cytogenetic methodology to identify increased number of gene loci with reference *CEP17* DNA sequence for accurate *HER2* assessment [12].

The aim of the present study was the clinical assessment of *HER2* proto-oncogene amplification and chromosome 17 copy numbers per cell by dual-probe fluorescence in situ hybridization (FISH) assay for demonstration of *HER2* protein overexpression in correlation with clinicopathologic parameters in the subgroup of IHC-equivocal (borderline 2+) patients for better selection of anti-*HER2* therapy.

## Materials and methods

### Patients/specimen selection

A cohort of 154 IHC-equivocal (2+) cases of female invasive breast carcinoma patients were selected between 2012 and February 2014 from histopathology tissue bank of Shifa International Hospital (SIH), Pakistan (including both in-hospitalized and referral cases). The paraffin-embedded tissue specimens immunohistochemically diagnosed as weak *HER2* oncoprotein overexpression were included in the study and retrospectively analyzed by dual-probe FISH assay for the clinical assessment of *HER2* oncogene and aneusomy 17 in the Division of Histopathology and Cytogenetic Pathology Lab at SIH in collaboration with Cancer Genetics Lab of Quaid-I-Azam University, Islamabad, Pakistan. The study

protocol was approved by the Institutional Review Board and Ethical Committee (IRB-EC) of SIH and Shifa College of Medicine (SCM) with a reference number (IRB-EC #2069-118-2013).

### Clinicohistopathological and immunohistochemical parameters

Patient's age, sex, and histopathological parameters including microscopic tumor size, histological type and grades, pathological stage, lymph-vascular invasion, and ductal carcinoma in situ (DCIS) were noted for each case. All the selected cases were immunohistochemically proven for *HER2* oncoprotein (2+) at borderline stage and estrogen and progesterone hormone receptor statuses (ER and PR) were also noted for each case.

### Dual-probe fluorescence in situ hybridization assay

FISH assay was performed on tissue specimens using the FDA-approved PathVysion *HER2* DNA Probe kit (Abbott Molecular Inc., Des Plaines, IL, USA), composed of two probes: LSI *HER2* (locus-specific identifier *HER2*) Spectrum Red fluorophore-labeled DNA probe for the *HER2* gene locus (17q12–21.32) to determine *HER2* gene amplification status and *CEP17* (centromere enumeration probe 17) Spectrum Green fluorophore-labeled alpha satellite DNA sequence probe for chromosome 17 to enumerate copy number of chromosome 17 per cell. The FISH procedure was carried out according to kit manufacturer instructions. The tissue sections of 4–6- $\mu$ m thickness were cut from formalin-fixed paraffin-embedded tissue blocks using rotary microtome and floated in a protein-free water bath at 40 °C to mount the sections on organosilane-coated slides. After air-dried, the slides were baked overnight at 56 °C. The slides were deparaffinized in Hemo-De for 10 min at room temperature, dehydrated in 100 % ethanol for 5 min at room temperature, and air-dried. Subsequently, the slides were pretreated with protease K solution at 37 °C for 10 min. Denaturation of specimen DNA was done in humidified hybridization chamber at 72 °C for 30 min and dehydrated the slides through ethanol gradients 70, 85, and 100 %. The denatured DNA specimen was hybridized with pre-warmed probes mixture on a 45–50 °C slide-warmer for 2–5 min, covered with cover slip, and further incubated at 37 °C overnight, followed by post-hybridization treatment with wash buffer (2 $\times$  SSC/0.3 % NP-40), and finally, slides were counterstained with DAPI. The slides were visualized under fluorescence microscope (Olympus, USA) and fluorescent probe signals were recorded in a minimum of 20 selected tumor nuclei for each case using CCD camera (Olympus) to enumerate *HER2* gene copies (red signals) and chromosome 17 copies (green signals). In case of variable result, the assay was repeated. Subjective

interpretations of FISH results were done by cooperation of pathologists and the average copy numbers and ratio of *HER2* (red signals) and CEP17 (green signals) were calculated in the fields containing invasive tumor component with non-overlapping tumor nuclei for the assessment of *HER2* gene amplification and chromosome 17. ASCO/(CAP) guidelines were followed for interpretation of *HER2* gene status [12].

### *HER2* gene interpretation criteria

| Average <i>HER2</i> copy number (signals/cell) | <i>HER2</i> /CEP17 ratio | <i>HER2</i> gene status      |
|--|--------------------------|------------------------------|
| ≥4 or <4                                       | ≥2                       | Amplified                    |
| ≥6   | <2                       | Amplified                    |
| ≥4 to <6                                       | <2                       | Equivocal (FISH borderline)* |
| <4   | <2                       | Non-amplified                |

\*Must order a reflex test (same specimen using IHC), test with alternative ISH chromosome 17 probe, or order a new test (new specimen if available, ISH or IHC)

Chromosome 17 aneusomy was determined by calculating an average CEP17 fluorescent green signals per cell nucleus and defined as CEP17 copies ≥3 (polysomy 17), CEP17 copies 1.5–2.5 (disomy), and CEP17 copies ≤1 (monosomy 17) [13].

### Statistical evaluation

SPSS statistical program software (version 16.0, IBM Chicago, IL, USA) was used to analyze the data. The results of *HER2* gene amplification by FISH method were correlated with aforementioned clinical and histological parameters using chi-square test with 95 % confidence interval;  $p < 0.05$  was considered significant.

### Ethical aspects

The study protocol, consent, and quaternary forms were approved by the Institutional Review Board and Ethical Committee (IRB-EC) of SIH and SCM, Islamabad, Pakistan. Reference of approval was IRB #2069-118-2013. All collected information including patient names and medical record numbers were handled anonymously.

## Results

### Biostatistics features

We studied 154 invasive breast carcinoma cases; all had *HER2* protein overexpression with 2+ score by IHC assay. In a cohort of 154 cases, the patient's age ranged between 33 and 93 years (mean, 53.04 years and mode 58, Std. Dev. ±11.43) with a majority of occurrence above 50 years. The majority of the cases were invasive ductal carcinomas (IDC; 132 cases, 85.71 %) and the rest were invasive lobular carcinoma (ILC; 22 cases, 14.28 %).

### Dual-probe fluorescence in situ hybridization evaluation

Dual-probe FISH assay was performed for *HER2* gene amplification (red signals) and chromosome 17 copy number (CEP17 green signals) per tumor cell. In a total of 154 cases, 36 cases (23.4 %) had *HER2* gene amplification (average *HER2* copies/cell ≥4 with *HER2*/CEP17 ratio >2), 114 (74.0 %) cases non-amplified (average *HER2* gene copies <4 with *HER2*/CEP17 ratio <2), and 4 cases (2.6 %) equivocal (average *HER2* copies 4–6 with *HER2*/CEP17 ratio <2) as shown in Table 1 and Fig. 1.

Of the 154 cases, a total of 18 cases (11.7 %) showed polysomy 17 (average CEP17/cell copies >3) in non-amplified, while more 13 cases (8.4 %) and 9 cases (5.8 %) presented monosomy 17 (average CEP17/cell) in amplified and non-amplified cases, respectively. One hundred fourteen cases (74 %) had disomy with *HER2* gene status distributed as 23 cases (14.9 %), 4 cases (4.6 %), and 87 cases (56.5 %) in FISH-amplified, equivocal, and non-amplified, respectively, and significant correlation between aneusomy 17 and *HER2* gene amplification was observed as  $p < 0.001$  (Table 1 and Fig. 1).

In Fig. 1a, the FISH results show *HER2* (red) and CEP17 (green) probe signals for detection of an average *HER2* gene and chromosome 17 copy numbers per cell in invasive breast carcinoma by dual-probe FISH. In panel a, average *HER2* copies 4> and *HER2*/CEP17 >2.86 indicated *HER2* gene amplification (×1,000). In panel b, *HER2* copies 2.05 and *HER2*/CEP17 >1.9 FISH-equivocal. In panel c, *HER2* copies 3 with ratio 1.5 presenting non-amplification. In panel d, CEP17 green signals counted with an average CEP17 copy number/cell nucleus found 3.4 greater than >3 indicated presence of polysomy 17; in panel e, green signals/cell nucleus found 0.8 lesser than <1 indicated monosomy; and in panel f, green signals/cell were 1.53 that is in the range of 1.5–2.5 demonstrated as disomy (normal).

**Table 1** FISH results for *HER2* gene and chromosome 17 status

|             |             | Total <i>n</i> (%) | <i>HER2</i> gene amplification <i>n</i> (%) |           |               | <i>p</i> value |
|-------------|-------------|--------------------|---|-----------|---------------|----------------|
|             |             |                    | Amplified                                   | Equivocal | Non-amplified |                |
| Aneusomy 17 | Polysomy 17 | 18 (11.7)          | 0 (0)                                       | 0 (0)     | 18 (11.7)     | <0.001         |
|             | Monosomy 17 | 22 (14.3)          | 13 (8.4)                                    | 0 (0)     | 9 (5.8)       |                |
|             | Disomy 17   | 114 (74)           | 23 (14.9)                                   | 4 (2.6)   | 87 (56.5)     |                |
| Total       |             | 154                | 36 (23.4)                                   | 4 (2.6)   | 114 (74)      |                |

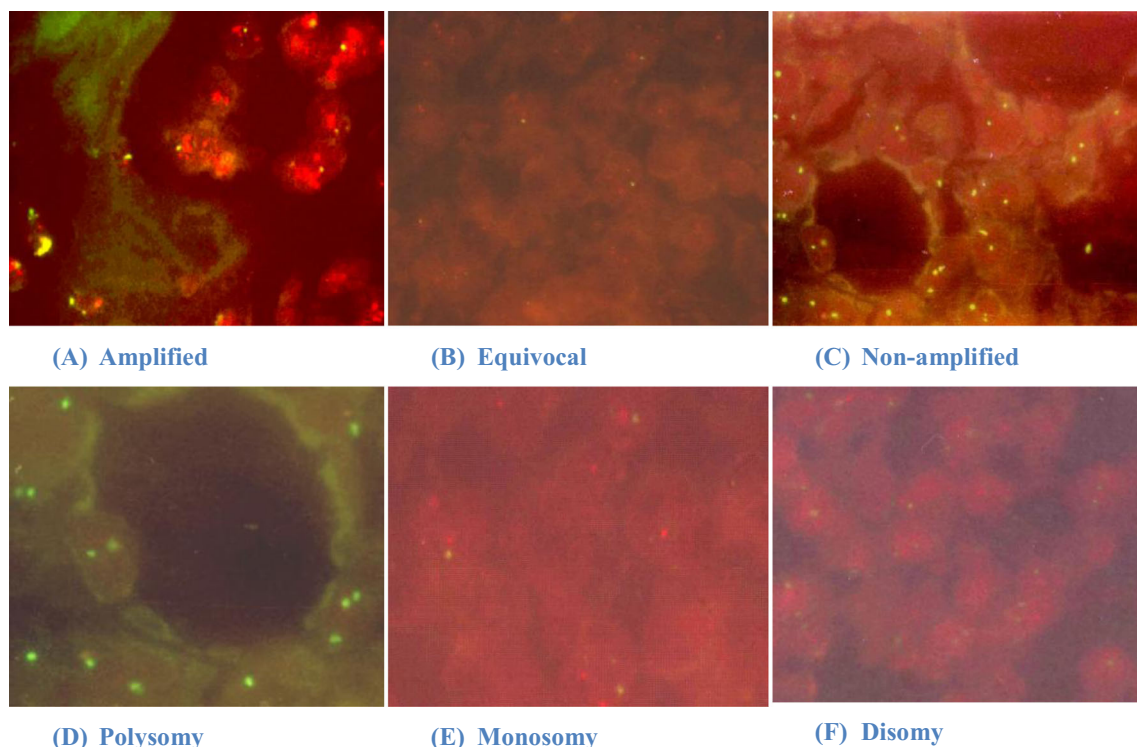
*HER2* human epidermal growth receptor 2

#### Frequency of *HER2* gene amplification in correlation with estrogen and progesterone receptors

Of the 154 IHC2+ cases, 128 (83.1 %) cases were estrogen receptor-positive (ER+) and 83 (53.9 %) cases were progesterone receptor-positive (PR+); among these, 20 cases (13.0 %) showed relatively high and 6 cases (3.9 %) showed lower *HER2* gene amplification, respectively. On the other hand, 26 (16.9 %) ER-negative and 71 (46.1 %) PR-negative cases showed the frequency of gene amplification as 16 (10.4 %) and 30 (19.5 %), respectively, which demonstrated that negative cases of either ER or PR had more gene amplification frequency as compared to ER- or PR-positive cases. This difference is highly correlated and statistically significant ( $p < 0.001$ , Table 2).

#### Association of *HER2* gene amplification with clinicohistopathologic variables

An excellent correlation was found between gene amplification and prognostic factors in borderline (IHC 2+)/*HER2* oncoprotein overexpressed cases. It was clear that *HER2* gene amplification was more frequent in older patients, ductal carcinoma, high-grade tumors, and hormone receptor-negative tumors, but no significant difference was observed in other factors. It was found that incidence of *HER2* gene amplification ( $n = 20$  (13.0 %)) is more in older >50 years patients than <50 years ( $n = 16$  (10.4 %)) but correlation was nonsignificant ( $p = 0.072$ ). IDC ( $n = 132$  (85.7 %)) patients had a gene amplification frequency in 36 cases (23.45 %) versus ILC patients ( $n = 22$  (14.3 %)) presented no case of



**Fig. 1** FISH results showing *HER2* (red) and CEP17 (green) probe signals

**Table 2** Correlation of FISH-based *HER2* gene amplification with expression of IHC-based estrogen and progesterone reception

| <i>HER2</i> gene status | ER         |           | Total <i>N</i> (%) | <i>p</i> value | PR        |           | Total <i>N</i> (%) | <i>p</i> value |
|-------------------------|------------|-----------|--------------------|----------------|-----------|-----------|--------------------|----------------|
|                         | Positive   | Negative  |                    |                | Positive  | Negative  |                    |                |
| Amplified               | 20 (13.0)  | 16 (10.4) | 36 (23.4)          | <0.001         | 6 (3.9)   | 30 (19.5) | 36 (23.4)          | <0.001         |
| Non-amplified           | 104 (67.5) | 10 (6.5)  | 114 (74)           |                | 77 (50)   | 37 (24)   | 114 (74)           |                |
| Equivocal               | 4 (2.6)    | 0 (0.0)   | 4 (2.6)            |                | 0         | 4 (2.6)   | 4 (2.6)            |                |
| Total <i>N</i> (%)      | 128 (83.1) | 26 (16.9) | 154                |                | 83 (53.9) | 71 (46.1) | 154                |                |

gene amplification; thus, a significant correlation was found between histological type and gene amplification as  $p=0.011$  (Table 3).

Microscopic tumor size recorded in 154 cases had been characterized between less than  $\leq 2$  cm ( $n=33$  (21.4 %)) and greater than  $>2$  ( $n=121$  (78.6 %)), of these, 12 (7.8 %) while 24 (15.6 %) showed gene amplification, respectively; the correlation of microscopic tumor size and gene amplification was nonsignificant ( $p=0.094$ , Table 3).

Seventy-seven cases (50 %) were high grade (grade III), 65 (42.2 %) were grade II, and 12 (7.8 %) were of low grade (grade I). The frequency of gene amplification was then determined for clinical stages I–III, for which the rates were observed as 0 %, 13 % (8.4 %), and 23 % (14.9 %) *HER* gene amplification, respectively. Thus, it appeared from the data that the rate of gene amplification is more in clinical stage III while decreased rate found in grade II with 2.6 % gene amplification at a cutoff point (equivocal) and non-amplified

**Table 3** Association of *HER2* gene amplification with clinico-pathological variables

| Parameters            | Total <i>n</i> (%) | <i>HER2</i> gene amplification |           |               | <i>p</i> value |
|-----------------------|--------------------|--------------------------------|-----------|---------------|----------------|
|                       |                    | Amplified                      | Equivocal | Non-amplified |                |
| Age (years)           |                    |                                |           |               | 0.072          |
| $\leq 50$             | $n=68$ (44.2)      | 16 (10.4)                      | 4 (6.2)   | 48 (31.2)     |                |
| $>50$                 | $n=86$ (55.8)      | 20 (13.0)                      | 0 (0)     | 66 (42.9)     |                |
| Microscopic size (cm) |                    |                                |           |               | 0.094          |
| $\leq 2$              | $n=33$ (21.4)      | 12 (7.8)                       | 0 (0.0)   | 21 (13.6)     |                |
| $>2$                  | $n=121$ (78.6)     | 24 (15.6)                      | 4 (2.6)   | 93 (60.4)     |                |
| Histological type     |                    |                                |           |               | 0.011          |
| IDC                   | 132 (85.7)         | 36 (23.4)                      | 4 (2.6)   | 92 (59.7)     |                |
| ILC                   | 22 (14.3)          | 0 (0)                          | 0 (0)     | 22 (14.3)     |                |
| Histological grade    |                    |                                |           |               | 0.024          |
| I                     | 12 (7.8)           | 0 (0.0)                        | 0 (0.0)   | 12 (7.8)      |                |
| II                    | 65 (42.2)          | 13 (8.4)                       | 4 (2.6)   | 48 (31.2)     |                |
| III                   | 77 (50.0)          | 23 (14.9)                      | 0 (0.0)   | 54 (35.1)     |                |
| Pathologic stage      |                    |                                |           |               | 0.001          |
| pT0                   | $n=4$ (2.6)        | 4 (2.6)                        | 0 (0)     | 0 (0)         |                |
| pT1                   | $n=19$ (12.3)      | 7 (4.5)                        | 0 (0)     | 12 (7.8)      |                |
| pT2                   | $n=114$ (74.0)     | 17 (11)                        | 4 (2.6)   | 93 (60.4)     |                |
| pT3                   | $n=9$ (5.8)        | 4 (2.6)                        | 0 (0)     | 5 (3.2)       |                |
| pT4                   | $n=8$ (5.2)        | 4 (2.6)                        | 0 (0)     | 4 (2.6)       |                |
| Lymphatic invasions   |                    |                                |           |               | 0.043          |
| Absent                | 72 (46.8)          | 13 (8.4)                       | 0 (0.0)   | 59 (38.3)     |                |
| Present               | 82 (53.2)          | 23 (14.9)                      | 4 (2.6)   | 55 (35.7)     |                |
| Vascular invasion     |                    |                                |           |               | 0.152          |
| Absent                | 114 (74.0)         | 29 (18.8)                      | 4 (2.6)   | 81 (52.6)     |                |
| Present               | 40 (26.0)          | 7 (4.5)                        | 0 (0.0)   | 33 (21.4)     |                |
| DCIS                  |                    |                                |           |               | <0.001         |
| Absent                | 99 64.3            | 13 (8.4)                       | 4 (2.6)   | 82 (53.2)     |                |
| Present               | 55 35.7            | 23 (14.9)                      | 0 (0)     | 32 (20.8)     |                |

cases were  $n=54$  (35.1 %). Our results show that the rates of gene amplification tumors in IHC 2+ cases of patients were significant ( $p=0.024$ ; Table 3).

The majority of pathological staged pT2 cases ( $n=114$  (74.0 %)) showed that a gene amplification 17 (11 %) relatively had a higher rate than the rest of the stages such as pT0, pT1, pT3, and pT4 ( $p=0.024$ , Table 3).

Among prognostic markers Lymphatic-positive tumors  $n=82$  (53.2 %) had gene amplification  $n=23$  (14.9 %) while DCIS-positive tumors  $n=55$  (35.7 %) showed gene amplification  $n=23$  (14.9 %). Prognostic markers presented significant correlations for gene amplification with  $p$  values of 0.043 and  $<0.001$ , respectively, compared to non-significant vascular involvement with  $p=0.152$  (Table 3).

## Discussion

Overexpressed HER2 protein is a potent predictive and prognostic biological marker in invasive breast carcinomas, but its accurate diagnostic evaluation by IHC method has been considered uncertain particularly in subgroup showing IHC 2+ score (IHC-equivocal) [14]. In the present study, we investigated HER2 gene amplification and chromosomal 17 aneusomy for true molecular assessment of HER2 status cytogenetically using dual-probe FISH assay in a cohort of IHC-equivocal cases of invasive breast cancer patients.

*HER2* gene amplification has been reported in numerous studies in the range of 18–20 to 25–30 % of invasive breast tumors as a whole [15] but concordance between HER2 protein overexpression and gene amplification drastically varies that is very low in IHC-negative (0/+1) to high in IHC-positive (3+) cases; however, IHC-equivocal (2+) cases have heterogeneous observations. According to the new HER2 guidelines from American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) [12] for the interpretation of FISH results, we observed 23.4 % HER2 gene amplification identification (average gene copies  $>4$  with *HER2*/CEP17 ratio  $>2$ ) in paraffin-embedded tissue specimens showing that IHC2+/FISH+ indicated breast tumor is true HER2-positive and patients with these findings should be subjected for anti-HER2 treatment. In previous studies, 6–25 % incidence of IHC 2+/FISH amplified cases had been reported [16, 17].

In a study, 28 out of 90 had IHC-equivocal (2+) cases and showed 17.8 % FISH-equivocal cases [18] but standard ASCO/CAP guidelines recommend that FISH-equivocal results should be less than 3 % in all specimens to ensure better FISH procedure [17]. In our study, four cases (2.6 %) were FISH-equivocal (*HER2* gene copies range between  $\geq 4$  and  $>6$ ) indicating more reliability in FISH procedure and interpretation and support the new ASCO/CAP recommendations

for the accurate evaluation of true gene amplification on chromosome 17. Among this subset of patients, further studies for the validation and new criteria are needed for better interpretations of such cases in order to get potential benefits from anti-HER2 therapy and to avoid any potential risk of chemotherapeutic agents. Thus, all breast cancer patients with HER2 IHC score of 2+ should be recommended for HER2 gene amplification assay for molecular genetic testing using dual-probe FISH analysis.

Interestingly, FISH non-amplified cases presented a secondary alternative mechanism of polysomy 17 for increase *HER2* gene copies resulting by extra gain of chromosome 17 (as defined  $\geq 3$  CEP17 copies/cell). In the present study, aneusomy 17 was observed and CEP17 probe (centromere enumeration probe) plays the key role to identify chromosome 17 aneusomy and is helpful to resolve such chromosomal genetic discrepancies in this subgroup of breast carcinoma with HER2 protein weak overexpression in non-amplified cases. Breast carcinomas harbor the frequency range from 10 to 50 % of polysomy in all IHC cases (negative 0, 1+, 2+, 3+) [17, 19]. Numerous studies have also postulated that it is more likely that the weak HER2 protein overexpression in non-amplified cases might be resulting from polysomy 17, an unconventional mechanism to increase the additional copies of HER2 gene per cell nucleus [17, 20]. In the ASCO/CAP guidelines, approximately 8 % equivocal cases had the evidence of polysomy 17 [17], while in our study, 11.7 % IHC-equivocal (2+) breast tumors (18 out of 154) presented polysomy 17 cases (CEP17 copies  $\geq 3$  per cell) in 74 % unamplified invasive breast cancers associated with adverse histopathological biomarkers such as histological grade III, advanced DCIS, and negative ER and PR receptors.

However, polysomy 17 has been found both in amplified and non-amplified tumors; more frequently observed are the non-amplified cases and our findings support these observations [13]. The occurrence of chromosome 17 polysomy is not rare in breast tumors and it has been suggested to explain the HER2 protein overexpression without true gene amplification that is falsely interpreted as positive HER2 protein overexpression in IHC analysis [18] but molecular analysis by dual-probe FISH assay resolves this discrepancy that the weak HER2 protein expression may be caused by extra gain of chromosome 17. Yet, insufficient data has been published to recommend potential benefit from anti-HER2 therapy in this genetically heterogeneous subclass. In this study, polyploidy phenomenon was explained by using CEP17 probe that reflects the number of chromosome copies per cell; this is particularly important where FISH-*HER2*/CEP17 ratios are  $<2$  but the *HER2* gene copy numbers  $>4$  per cell.

Single-gene overexpression phenomenon had been observed in tumor cell harboring single chromosome 17 copy

per cell (monosomy 17, less than two copies of CEP17 signals per cell) than normal state (disomy, two copies per cell) [19]. Various studies have been reported to address these variabilities and about 10 % of these alterations have been proposed to be caused by gene overexpression at the mRNA transcriptional level or at posttranslational levels [21]. In our study, we found that in nine cases (5.8 %) HER2 protein overexpression might be caused by single-gene copy overexpression reflected by decreased CEP17 probe signals per cell ( $CEP17 \leq 1$ ), thus exploring and elaborating the phenomenon of *HER2* gene overexpression and not gene amplification in this subset of breast carcinoma patients.

Thirteen percent ER+ and 19 % PR-negative receptors showed a significant correlation with *HER2* gene amplification in the present study, relating to near similar observations in previous studies like 16 % cases showed both expression of ER and *HER2* amplification [15, 22, 23]. The relation between hormone receptor and *HER2* amplification is proposed to be complex interactive signaling pathway in breast cancer cells [24].

In the present study, there is no statistically significant correlation of *HER2* gene amplification with microscopic tumor size and age; however, patients aged greater than 50 years present more occurrences. Fifty percent high-grade (III) breast tumors cases had 14.9 % gene amplification relatively high than low grades (I and II) which is concordant with the previous studies [18, 25]. Previous studies correlated with ductal carcinomas in situ (DCIS) had a high proportion of gene amplification, while our study data presented gene amplification in almost all cases of invasive breast cancer (IDC) [18]. Pathological factors including pathological type (pT2) and positive lymphatic invasion show significant correlation with *HER2* gene amplification in the present study.

In case of 23.4 % *HER2* gene amplification cases of invasive breast carcinoma patients, distinguished with protein overexpression evaluation by dual-probe FISH assay, targeting of *HER2* receptor with adjuvant herceptin therapy can be used as a better approach. Our findings support to interpret and distinguish the weak *HER2* oncoprotein overexpression in ambiguous heterogeneous nature of invasive breast tumors because they could either be resulting from *HER2* oncogene amplification (multiple *HER2* gene amplicons on same chromosome 17), polysomy 17 (increased *HER2* gene copies by extra gain of chromosome per cell) mostly in FISH non-amplified cases, or by monosomy 17 (single *HER2* gene overexpression). The data provides information that combined *HER2* assessment at protein and gene level is quite vital for the recruitment of anti-*HER2* treatment eligibility and to rule out false positive and negative IHC results to avoid the potential chemotherapy risks in this subgroup.

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