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Evaluation of HER2 gene amplification status in invasive breast cancer patients by Fluorescence in Situ Hybridization analysis and its correlation with clinical features

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A B S T R A C T

Precise assessment of HER2 gene status as an important biomarker plays a significant role in identifying the eligible patients for Trastuzumab therapy and determining their clinical outcomes. In this study, the researchers assigned HER2 amplification status in invasive breast cancer specimens by Fluorescence in Situ Hybridization (FISH) and determined its association with other clinical features. Formalin-fixed paraffin embedded tumor tissue specimens of 46 patients with invasive breast cancer were collected from November 2011 till May 2012. HER2status was evaluated by FISH. The Zytolight SPEC HER2/CEN17 dual color probe kit was applied for assessment of HER2status. HER2 gene amplification was defined as HER2/CEP17 ratio>2.2.The association between HER2status and clinical features like tumor grade, tumor type, tumor size, axillary lymph node involvement and age of patient was done using Chi squared test at the 0.05 level of significance (*p* value). Amplification of HER2 gene was detected in twelve cases (26%). On statistical analysis HER2status showed correlation with tumor grade (p=0.02).There was no correlation between HER2status and tumor type, tumor size, lymph node status and age of patients. The results of this study are consonant with the findings of other studies about the presence of HER2 gene amplification in invasive breast cancer. Statistical analysis showed patients with HER2 amplified gene have tumors with higher grade. In these patients the probability of increased proliferation and metastasis is high therefore evaluation of HER2 gene amplification status in breast cancer patients specially in high grade tumor with an accurate method such as FISH is essential.

Key words: *Breast cancer, HER2 gene, gene amplification, Fluorescence in Situ Hybridization (FISH), clinical features*

Abbreviations: FISH- Fluorescence in Situ Hybridization

Introduction

Breast cancer is the second most common cancer after lung cancer in the world(Jemal et al., 2010) and is the fifth cause of cancer related death(Hutchinson, 2010)Breast cancers are classified based on several factors such as tumor grade, tumor stage, tumor type , tumor size, lymph node status and others (Alizart et al., 2012). The biomarkers used for breast cancer classification include estrogen receptor (ER), progersterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) (Fitzgibbons et al., 2000).Among these factors, the HER2 gene status or its protein expression has both a predictive and prognostic value (Henry and Hayes, 2006).The HER2 gene is located on chromosome 17q21 and its product is a transmembrane growth factor receptor . This receptor is involved in the cellular signaling regulating growth and development (Popescu et al., 1989). The HER2 protein over expression after gene amplification in breast cancer results in inordinate activation of the signaling pathways (Yarden, 2001). Increased HER2 activity leads to in resistance to conventional therapy (Colomer et al., 2007; Ross et al., 2009). Trastuzumab (Herceptin) is the most widely used therapeutic option in breast cancer patients with HER2 gene amplification(Ross et al., 2009). Trastuzumab is a recombinant humanized monoclonal antibody

against the extracellulardomain of HER2. Trastuzumab is useful only in patients with HER2 gene amplification or protein overexpression (Mass et al., 2005). Trastuzumab treatment in HER2 negative cancer cases associated with side effects such as cardiotoxicity (Moelans et al., 2011). Therefore accurate evaluation of HER2 status is important in treatment decision. Several methods are available for determination of HER2 protein overexpression or gene amplification (Penault-Llorca et al., 2009). HER2 status at protein level is assessed by immunohistochemistry (IHC), ELISA and Western blot.HER2 status at DNA level is determined by Southern blot, Slot blot, CISH, FISH, and MLPA. HER2 status assessment at RNA level is done by qRT-PCR and microarray (Moelans et al., 2011). Currently HER2 status is tested mostly by two methods: immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH). FISH is considered as a preferable technique comparison to IHC because, stability of DNA as a target is higher than the HER2 protein. In addition, FISH is a quantitative interpretation and is easier for interlaboratory standardization (Sauter et al., 2009). Based on the findings of several studies, FISH technique has been determined as the most precise method for detection of HER2 gene amplification and determination of response to HER2 targeted therapy (Mass et al., 2005). Our objective in the present study was to assign HER2 gene status in patients with invasive breast cancer by FISH. Secondly, we determined the correlation between HER2 gene amplification and clinical features like tumor grad, tumor type, tumor size, axillary lymph node involvement and age of patient.

Materials and Methods

The study used formalin fixed paraffin embedded tissue blocks from 46 histologically proven invasive breast cancer cases. These cases were collected from Dr. Faghihi laboratory in Isfahan from November 2011 till May 2012. This study was approved by the institutional ethics committee of Isfahan University. All patients were informed and they signed standardized written consent. Clinical data including tumor type, tumor grade, tumor size, lymph node status and age of patients, if possible, were provided for cases from file of patients in the hospital. Assessment of HER2 gene status was done by the Zytolight SPEC HER2/CEN17 dual color probe kit (ZytoVision, Bremerhaven, Germany). The probe contains green-labeled polynucleotides (ZyGreen: excitation at 547 nm and emission at528 nm) which target the HER2 gene and orange labeled polynucleotides (ZyOrange: excitation at 547 nm and emission at 572 nm) which target alpha-satellite-sequences of the centromere of chromosome 17. FISH procedure was performed according to the manufacture´s instruction. Subsequently, duplex formation of the fluorescent-labeled probe was assessed using Olympus BX5 florescent microscope (Olympus, Tokyo, Japan) fitted with suitable filters for spectrum orange, spectrum green and DAPI. The 4 µm sections were drown up onto silane-coated slides. Slides were incubated for 10 min at 70ºC on a hot plate and deparafinized for 2× 10 minutes in xylene (Merck KGaA, Darmstadt, Germany). After dehydration in decreasing concentration of ethanol (100%,100%, 90%, 70%) each for 5 minutes and washing in distilled water 2 × 2 minutes , the slides were immersed in pre-warmed heat pretreatment solution citric provided in the kit at 98ºC for 15 minutes then slides were washed for 2× 2 minutes in distilled water and drained off. Pepsin solution was applied to the tissue section and slides were incubated for 10 minutes in a humidity chamber. After enzymatic digestion the slides were rinsed in wash buffer SSC for 5 minutes and in distilled water for 1 minute and dehydrated in ethanol 70%, 90% and 100% each for 1 minute. Determination of digest degree was done by applying 10µl DAPI and slides were evaluated under florescent microscope. After pretreatment a ready-to-use dual color probe consisting of HER2 HER2/neu and chromosome 17 probe was applied. The slides were covered with a cover slip and heated for 10 minutes at 75ºC on a hot plate for denaturation. The slides were incubated overnight at 37ºC in a humidity chamber. Post hybridization washing was carried out followed by washing in 1x wash buffer provided in the kit for 2 x 5 minutes at 37ºC. Slides were dehydrated in graded series of ethanol (70%, 90%, 100%) each for 1minute and air dried. Then the slides were counterstained with 30µl DAPI. The FISH specimens were analyzed using by Olympus BX5 florescent microscope (Olympus, Tokyo, Japan). In each case the number of HER2 signals and CEP17 signals were enumerated in 40 morphologically intact and no overlapping nuclei. The ratio of the number of HER2 signals to the number of chr17 signals per nucleus was used to score (Penault-Llorca et al., 2009). According to ASCO/CAP guidelines ratio of HER2 to CEP17 of >2.2 was considered as HER2 gene amplification, FISH ratio of <1.8 was interpreted as negative and FISH ratio of 1.8-2.2 was considered equivocal (Wolff et al., 2006).

Statistical analysis: The association between HER2 status and clinical features like tumor grade, tumor type, tumor size, axillary lymph node involvement and age of patients was done using Chi squared test at the 0.05 level of significance (*p* value).

Results and Discussion

Gene amplification was evaluated in 46 formalin fixed paraffin embedded invasive breast cancer tissues by FISH. Twelve cases (26%) showed amplification of HER2 gene and in the rest of the samples (34 or74%) amplification was not detected. In the present study, there were no equivocal FISH results. Clinical and histological features of the patients are shown in Table 1. Based on Statistical analysis there was association between tumor grade and HER2status (*p* value=0.02) .HER2 gene amplification was observed in tumors with high pathological grade. While ductal carcinoma cases had HER2 gene amplification more frequently (83.3%) than other types of breast cancer, no significant correlation was seen between tumor type and HER2 status. HER2status was not associated with tumor size, lymph node status and age of patients. Clinically in

breast cancer, HER2 gene amplification has diagnostic and prognostic usefulness. The overexpression of the HER2 protein following the HER2 gene amplification results in increased breast cell proliferation, survival and motility, all of which can lead to the formation of a malignant breast tumor(Ross et al., 2009; Badache and Gonçalves., 2006). Human breast cancers with HER2 gene amplification are highly aggressive and resistant to traditional treatments(Slamon et al., 1987; Yu et al.,2000). Trastuzumab, a recombinant humanized monoclonal antibody, downregulates HER2 and inhibits HER2 induced signaling cascades hence, prevents proliferation of human breast cancer cells with amplified HER2 (Hudziak et al., 1987). Trastuzumab remarkably increases the survival of patients with HER2 positive cancer but despite the efficacy of trastuzumab, it has deleterious side-effects. Therefore accurate assessing of HER2 status for identifying patients whose tumors are amplified for the gene and respond toanti-HER2 therapy is indispensable for treatment decision. FISH and IHC are the most used techniques for HER2 status evaluation (Wolff et al., 2006). In terms of methodological and biological aspects, FISH technique is considered as a primary HER2 status testing in patients with breast cancer (Sauter et al., 2009). This technique is a rapid, accurate and reproducible method for HER2status assessment. Estimations about the proportion of HER2 gene amplification in breast cancer in different studies is in the range of 18%-30% (Wolff et al., 2006; Slamon et al., 1989). In the present study, 26% of patients had gene amplification; this is consonant with the results of other studies. In invasive breast cancer,HER2 amplification occurs at significantly higher level in ductal carcinomas than in lobular carcinomas (Hoff et al., 2002; Ariga et al., 2005) and HER2 amplification is known to have inverse correlation with the lobular tumor type (Bane et al., 2005). In this study, HER2 gene amplification was observed more frequently in cases with invasive ductal carcinoma tumors but there was no significant correlation between tumor type and HER2 status. Some prior studies have reported association between HER2 amplification and prognostic factors such as tumor size (Borg et al., 1990) and axillary lymph node involvement (Borg et al., 1990; Gusterson et al., 1992). In the present study, no correlation was observed between tumor size, lymph node status and HER2 gene amplification. These findings are concordant with reported results in existing literature (Panjwani et al., 2010; Prati et al., 2005). No significant relationship was found between HER2 gene amplification and age of patients as previous studies (Pinto et al., 2001; SezgÄN Ramadan et al., 2011). Panjwani et al and Yau et al have reported a correlation between HER2 amplification and tumor grade (Panjwani et al., 2010; Yau et al., 2008). Statistical analysis in this study also showed concordance between HER2status and tumor grade. It has been showed that 97% of cases with HER2 gene amplification have grade II and III.

In conclusion, the result of this study like the findings of other studies showed presence of HER2 gene amplification in invasive breast cancer. Also, the association between HER2 gene amplification and tumors with high pathological grade indicate poor prognosis of this kind of tumors and supplication of HER2 assessment for efficient management of disease and selection of useful treatment.

Table 1. Clinical and histological characteristics of 46 invasive breast cancer patients

Characteristic	Number of patients (%)
Age (yr)	
≤50	30(65.2)
>50	16(34.8)
Tumor size(cm)	
<2	17(37)
≥2	20(43.5)
Uncertain	19(19.5)
Histological type	
Ductal	29(63)
Lobular	7(15.2)
Other	10(21.7)
Histological grade	
Grade 1	5(10.9)
Grade 2	19(41.3)
Grade 3	15(32.6)
Uncertain	7(15.2)
Lymph node status	
Positive	20(43.5)
Negative	14(30.14)
Uncertain	12(26.1)

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