

Comparative Analysis of HER-2/neu Amplification, Overexpression and Polysomy 17 in Patients with Metastatic Breast Cancer

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ABSTRACT

Amplification or overexpression of HER-2/neu oncogene is associated with poor prognosis and has been correlated with the response to trastuzumab treatment. Chromosomal copy number changes of chromosome 17 occurs in breast cancers and effect the copy number of HER-2/neu gene and consequently IHC score relatively. The aim of this study was to investigate the contribution of chromosome 17 polysomy to positive HER-2/neu immunostaining independent of HER-2/neu amplification using dual-color FISH technique on the paraffin-embedded tissues of metastatic breast cancers. Paraffin-embedded specimen of 32 metastatic breast cancer cases were analysed using dual-color FISH probes specific to HER-2/neu gene and chromosome 17. Results showed significant association between the cases with IHC 2+ and polysomy 17 ($p=0.011$), and IHC 3+ and HER-2/neu amplification ($p=0.023$). In conclusion, increased HER-2/neu gene copy number in accordance with polysomy 17 might lead to a strong protein overexpression in the metastatic breast cancer. Therefore FISH technique may also be more sensitive interpretation tool in cases with polysomy 17.

Key Words: HER-2/neu, Metastatic breast cancers, IHC, FISH

ÖZET

Metastik Meme Kanselerinde HER-2/neu Amplifikasyonu, Ekspresyonu ve Polizomi 17'nin Karşılaştırmalı Analizi

HER-2/neu onkogeninin amplifikasyonu yada artmış ekspresyonu kötü prognozla ilişkilidir ve trastuzumab tedavisine yanıtla arasında korelasyon vardır. Meme kanserlerinde 17. kromozomu da içine alan sayısal anomaliler görülmekte ve buna bağlı olarak HER-2/neu gen sayısı vegen ürününün immune boyanma pozitiflik değerinde de rölatif olarak değişimler olabilmektedir. Bu çalışmanın amacı da 17. kromozomdaki bu sayısal değişikliklerin Her-2/neu genine ait immunohistokimyasal pozitif boyanmalara katkısını değerlendirmektir. 32 adet metastatik meme kanserinin parafinize edilmiş dokuları, HER-2/neu genine ve 17. kromozoma özgü iki-renkli FISH problemleri kullanılarak analiz edildi. Çalışma sonuçları, IHC 2+ boyanma ile polizomi 17 arasında istatistiksel olarak anlamlı ilişki olduğunu ortaya çıkardı ($p=0.011$), ayrıca IHC3+ boyanma ile de Her-2/neu amplifikasyonu arasında anlamlı ilişki vardı ($p=0.023$). Sonuç olarak 17. kromozomunun sayısal artışına bağlı olarak HER-2/neu geninin kopya sayısındaki artma, protein ekspresyonunun da armasına neden olmaktadır, böylece, polizomi 17 olan vakalarda yalancı poitiflikleri önlemede FISH analizinin yapılması daha da önemli hale gelmektedir.

Anahtar Kelimeler: HER-2/neu, Metastatik meme kanseri, IHC, FISH

INTRODUCTION

Breast cancer is the most common cancer among women and metastases is the major cause of death (1). Some prognostic factors for early-stage breast cancer have been confirmed in a number of studies, however, there have been only a few studies suggesting prognostic factors in breast cancer patients with metastases (2-4). The HER-2/neu (c-erbB-2) gene, located on chromosome 17q21 region encodes a 185-kDa transmembrane glycoprotein that has intracellular tyrosine kinase activity via amplification or overexpression in approximately 20-35% of breast cancers. Amplification or overexpression of HER-2/neu is associated with poor prognosis and has been correlated with response to adriamycin chemotherapy (5-7). HER-2 protein provokes cell division and stimulates cell motility which facilitating development of metastasis in tumors. The trastuzumab, a humanized monoclonal antibody to the extracellular region of HER-2/neu protein, has provided a new treatment for women with metastatic breast cancer, stop signal transduction by inducing endocytosis and degradation of HER-2, thus it can block tumour cell growth (8,9). HER-2/neu expression or amplification analysis is usually performed in the primary tumor tissues, because biopsy from metastatic lesion could not be performed practically (4). Fluorescence in situ hybridization (FISH) and Immunohistochemistry (IHC) are the very useful tools to evaluate Her2/neu status in breast cancer. New technique, chromogenic in situ hybridization (CISH) similar to FISH, has been developed recently in which HER-2/neu DNA probe uses a peroxidase reaction instead of a fluorescent dye and the tissue can be visualized easily along with the amplification product (9). Numerous studies showed that there was strong correlation between Her-2/neu gene amplification and IHC 3+ staining, but not between HER-2/neu gene amplification and IHC 2+ /IHC 1+ (11, 12). Therefore IHC 2+ scores have sometimes been considered "false positive" if there were no Her-2/neu gene amplification with FISH (13). Chromosomal polysomy affecting chromosome 17 occurs in breast cancer, consequently, it increases the copy number of HER-2/neu gene, thus, IHC score may be interpreted as positive (14). Some studies showed that different chromosome arms had copy

number changes besides chromosome 17 in breast cancers (15, 16). These findings confirmed that IHC positive score due to polysomy 17 is not specific event if there were other chromosomal polysomies except for chromosome 17 in breast cancers (17, 18). Therefore, true evaluation of HER-2/neu amplification, overexpression and chromosome 17 status becomes a key role in the maximum management of patients with metastatic breast cancer with HER-2 positive tumor.

In the present study, we investigated the contribution of chromosome 17 polysomy to positive HER-2/neu immunostaining independent of HER-2/neu amplification using dual-color FISH technique on the paraffin-embedded tissues of metastatic breast cancers.

MATERIAL AND METHOD

FISH analysis

FISH analysis was performed on primer tumor tissues and none of the samples had received radiotherapy or chemotherapy before analysis. Five paraffin embedded normal tissues were analysed to determine cut-off levels for FISH anomalies. The probe mixture consisted of the Kit "PathVysion" which included centromeric region of chromosome 17 (labeled-green) and HER-2/neu gene locus (labeled-orange) (Vysis, Downers Grove, IL, USA). Tissue sections were dewaxed in xylene and rehydrated in ethyl alcohol. Deparaffinised slides were treated with 0.2 N HCl for 20 min at room temperature and with 8% sodium thiosulphate at 80 °C for 30 min then treated with a protein-digesting enzyme (0.5 % pepsin) at 37°C for 20 min. FISH probe and tissue sections were denatured at 73°C for 3 min, then 10 µl solutions containing chromosome 17 centromere /HER-2/neu locus specific probes were applied to the tissue sections at 37 °C for 16 h. After hybridization, slides were washed, nuclei were counterstained with 10 µl a 4, 6-diamidine, 2-phenylindol (DAPI). Slides were examined and images were captured on a Quips Imaging System (Applied Biosystem, UK) equipped with Nikon E 600 epifluorescence microscope and a filter set (triple; dapi/red/green, dual color; red/green, single red and single green, Vysis, USA).

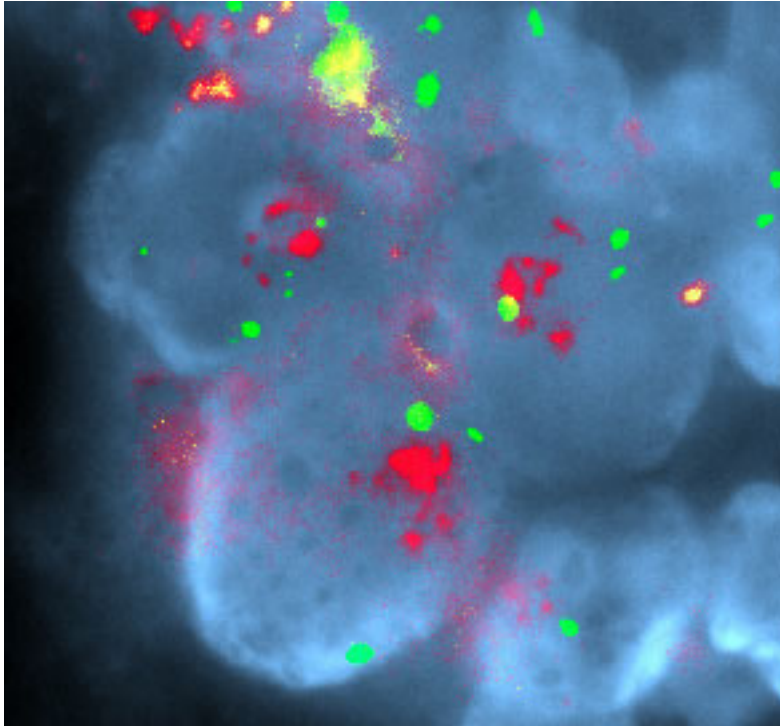


Figure 1. Representative example of Her-2/neu amplification; green signals show centromeric region of chromosome 17 and orange signals show Her 2/neu gene locus.

Scoring of FISH Signals

Dual color well-defined hybridization signals of chromosome 17 and HER-2/neu were countered within 100 interphase nuclei in different areas per specimen. Overlapping nuclei, disrupted nuclei, indistinguishable margins, and nuclei with indistinguishable signals were eliminated.

Immunohistochemistry

HER2_neu IHC staining was performed on 3-4 micron paraffin sections according to the manufacturer's instructions (monoclonal, HER-2/neu Ab-17 Clone e2-4001+3B5, Neomarkers). Citrat buffer and mikrowave antigen retrieval proces was used, incubation was made at 30 min in 1/600 dilution. Cell membrane staining was evaluated according to intensity as follows: no or detectable membrane signal in 10% of the tumour cells was considered as negative; weak membrane stain in of the tumour cells was scored as 1+ moderate to strong complete

membrane positivity in of the tumour cells was considered as 2+ and 3+, respectively.

Statistical Analysis

The association was investigated between IHC categorical results (neg, 1+, 2+, 3+) and polysomy 17 and Her2/neu amplification using "Mann Whitney" nonparametric test. Fisher's Exact test was used to investigate association between IHC, FISH results (for polysomy 17 and Her2/neu gene amplification) and metastatic organs (liver, lung, bone, lymph node and brain). Furthermore, we separated the IHC results as "negative" (neg and 1+) and "positive" (2+ and 3+), then we investigated the association between the IHC negative, positive results and polysomy 17, HER-2/neu amplification using Fisher's Exact test. The SPSS statistical software version 10.1 was used for the analyses. All p values were taken as significant at $p < 0.05$.

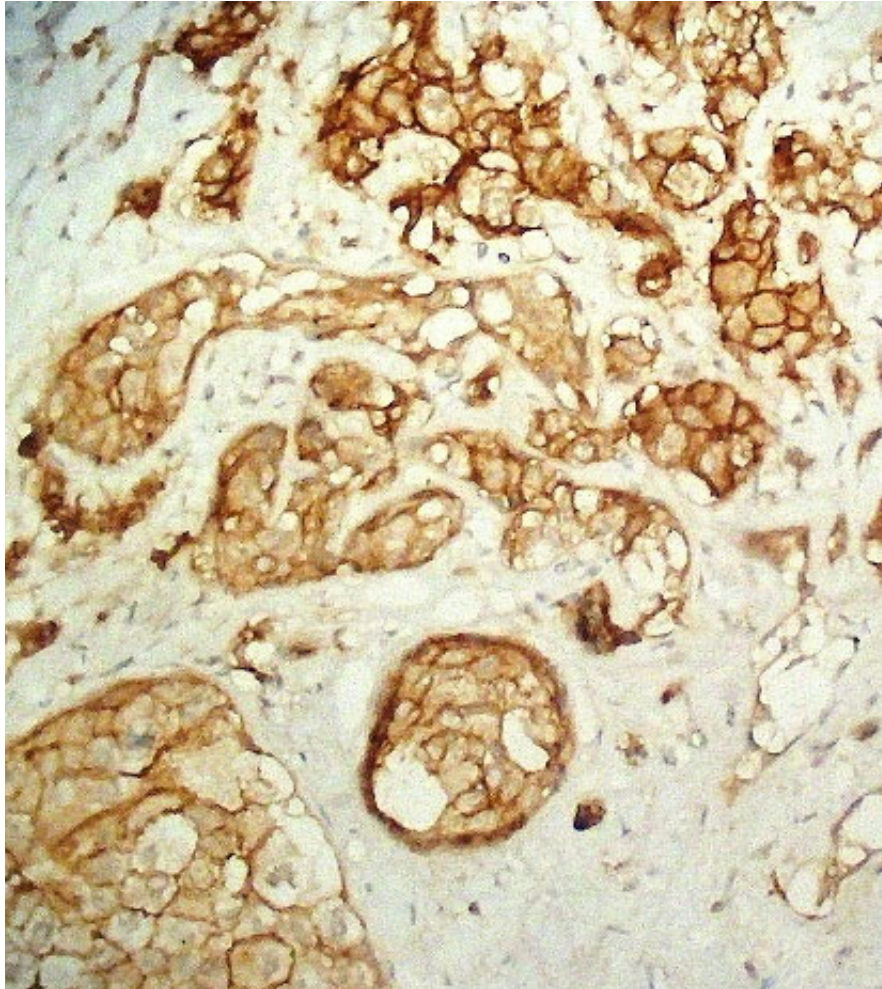


Figure 2. Representative examples of IHC 3+ images.

RESULTS

Paraffin embedded specimen of 32 cases diagnosed initially with metastatic breast cancer were analysed. The age of the cases ranged from 31 to 71 and the mean age was 50.6. The average copy number of HER-2/neu gene was $1.9 (\pm 0.2)$, the average copy number of chromosome 17 was $1.7 (\pm 0.2)$ in controls. Thus, HER-2/neu gene amplification was defined if signals of HER-2/neu / chromosome 17 ratio were greater than 2, polysomy 17 was assumed if signals of chromosome 17/cells were greater than 2. Among the 32 breast tumor samples, 9 had HER-2/neu amplification (28.1%) (Figure 1), 18 had polysomy 17 (56.2%). IHC analysis showed that 5 cases had negative staining, 8 had 1+, 11 had

2+ and 8 had 3+ staining. Among the 9 HER-2/neu amplified cases, 5 had IHC 3+ (Figure 2), 3 had IHC 2+ and one had IHC 1+ staining. Among the 9 HER-2/neu amplified cases, 6 had polysomy 17, 3 had disomy 17. The all observed variable genetic changes and clinical findings were presented in Table-1.

Statistical Analysis Results

The results of Mann Whitney analyses showed significant correlation between IHC categorical results and polysomy 17 ($p=0.001$) and between IHC categorical results and HER-2/neu amplification ($p=0.010$). Fisher's Exact test showed the relation-

Table 1. HER-2/neu and chromosome 17 status assessed by FISH and IHC in patients metastatic breast cancers.

Case	Age	Metastatic Organ	Her2/neu by IHC	Her-2/neu by FISH	Polysomy 17 by FISH
1	71	Liver	Neg	Neg	Neg
2	64	Liver	3+	Neg	Positive
3	48	Liver, Bone	2+	Positive	Positive
4	54	Brain	Neg	Neg	Neg
5	34	Liver	2+	Neg	Positive
6	52	Lymph Node	2+	Neg	Positive
7	34	Lung	3+	Positive	Positive
8	41	Liver	Neg	Neg	Neg
9	50	Liver, Lung	1+	Positive	Neg
10	60	Bone	2+	Neg	Positive
11	58	Liver	3+	Positive	Neg
12	41	Lymph Node	1+	Neg	Neg
13	55	Liver, Lung	3+	Positive	Neg
14	42	Bone	1+	Neg	Positive
15	49	Liver, surrenal	Neg	Neg	Neg
16	51	Lymph Node	3+	Neg	Positive
17	52	Lymph Node	2+	Neg	Positive
18	45	Bone	3+	Neg	Positive
19	64	Lymph Node	3+	Positive	Positive
20	31	Lymph Node	2+	Positive	Positive
21	43	Bone	Neg	Neg	Neg
22	50	Liver	1+	Neg	Neg
23	55	Lung	3+	Pozitive	Positive
24	62	Lung	1+	Neg	Neg
25	62	Lymph Node	2+	Pozitive	Positive
6	51	Liver	2+	Neg	Positive
27	63	Liver, Lung, Periton	2+	Neg	Positive
28	37	Lymph Node	2+	Neg	Positive
29	52	Lymph Node	1+	Neg	Neg
30	47	Bone	1+	Neg	Positive
31	53	Liver	2+	Neg	Neg
32	50	Lymph Node	1+	Neg	Neg

ship between the cases with IHC 2+ and cases with polysomy 17 ($p=0.008$). Fisher's Exact test also showed the relationship between the cases with IHC 3+ and cases with Her2/neu amplifications ($p=0.023$).

DISCUSSION

In this study Her2/neu amplification and protein expression and the effect of polisomy 17 on Her2/neu gene status were evaluated in metastatic breast cancers by FISH and IHC analyses. The four known genes TP53, ERBB2 (Her2/neu), BRCA1, and NM23 which play important role in breast can-

cer pathogenesis are located on chromosome 17 (19). Bossuyt et al. showed that the human epidermal growth factor receptor (EGFR) is overexpressed in breast cancers and has relation with squamous differentiation (20). Another study showed GRB7 and MLN64 expressions and their diagnostic value besides HER-2/neu in human breast cancer (21). PTEN pathway may have a role for the anti-tumor activity of trastuzumab in metastatic breast cancer patients (22).

In most of studies, the potential effect of copy number changes of chromosome 17 on HER-2/neu status was evaluated (18, 23, 24) and some of them revealed the association between prognosis and copy number changes (17,25,26). Variable copy number changes of chromosome 17 may effect the tumoral development by producing heterogeneity in tumor cells and this is the result of difference of tumor tissues in each case. Polisomy of chromosome 17 independent of HER-2/neu amplification is an important point.

The results of IHC analysis of 32 cases showed that 5 had IHC negative, 8 had IHC 1+, 11 had IHC 2+ and 8 had IHC 3+ which 59.3% of the cases had strong protein overexpression (11 cases with IHC2+ + 8 cases with IHC3+) (Table 1). At the end of the FISH analysis 9 cases had gene amplification (28.1%) and 18 cases had polysomy 17 (56.2%). None of the IHC negative cases showed polysomy 17 or HER-2/neu amplification which revealed strong concordance between IHC and FISH negativity. Two of 8 IHC 1+ cases showed polysomy 17 and one showed HER-2/neu amplification; there no concordance between IHC1+ and polysomy 17 or HER-2/neu amplification. Nine of 11 IHC 2+ cases showed polysomy 17 and 3 showed HER-2/neu amplification. There were appeared strong concordance between IHC2+ and polysomy 17. Six of 8 IHC 3+ cases showed polysomy 17 and 5 showed HER-2/neu amplification which showed strong concordance between IHC3+ and HER-2/neu amplification (Table 1). Results of statistical analysis revealed a significant association between protein overexpression and polysomy 17 ($p=0.001$) supporting our proposal. Furthermore, results showed significant association between IHC staining and Her2/neu amplification ($p=0.01$) signifying the concordance between IHC3+ and HER-

2/neu amplification. According to these results we suggest that independent of the HER-2/neu DNA amplification, polisomy 17 strongly effects the IHC staining relatively. Other studies revealing higher percentage of protein overexpression and lower HER2/neu amplification confirm this suggestion (13, 14, 27-30).

The discrimination of HER-2/neu amplification and polysomy 17 has important role in the event of improving more effective chemotherapy, because, the HER-2/neu gene is a viable therapeutic target for trastuzumab (31). In clinical trials, trastuzumab in combination with standard chemotherapy demonstrated improvement in outcome for breast cancer with HER-2/neu amplification. (14, 30). Therefore if patients have polysomy 17 with or without HER-2/neu amplification, clinicians can take into consideration other tumor suppressor genes and oncogenes on chromosome 17. Statistical analysis results supported this data showing strong correlation between the cases with IHC 3+ and with Her2/neu amplification ($p=0.023$) but did not show correlation between IHC negative, IHC1+, IHC2+ and Her2/neu amplification. The patients with overexpression of IHC 2+ and IHC1+ failed to show FISH gene amplification and may not benefit from the trastuzumab treatment. Paralel to our study, one study revealed association between strongly positive IHC3+ and gene amplification in breast cancer patients (32). Statistical analysis also showed the relationship between the cases with IHC 2+ and with polysomy 17 ($p=0.008$). So, especially the IHC 2+ scores can be considered positive if there is polysomy 17 in cases without Her-2/neu gene amplification. One study showed IHC 2+ and "false positive" relationship for Her-2/neu gene amplification (13). Fisher's Exact test results supported this data that showed significant correlation between the all IHC positive results and polysomy 17 ($p=0.001$) and showed border-line correlation between the all IHC positive results (1+, 2+, and 3+) and Her2/neu amplification ($p=0.05$). Therefore, true evaluation of HER-2/neu status using FISH method is needed and becomes important if there is IHC 2+ staining.

In conclusion, the present data demonstrated that polysomy 17 increases IHC2+ and IHC3+ staining, regardless of Her2/neu amplification in higher

percentages than that in IHC negative or IHC1+ staining tumors. Our data also showed that increased copy number of chromosome 17 was statistically associated with the IHC2+ staining and HER-2/neu gene amplification was statistically associated with the IHC (3+) staining, it will be cause to examine whether these IHC positive and FISH negative tumors with polysomy 17 are pathologically different from other IHC negative and FISH-negative tumors, especially in terms of response to trastuzumab therapy.

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