TÜBİTAK

Original Article

Turk J Med Sci
2011; 41 (5): 809-819
© TÜBİTAK
E-mail: medsci@tubitak.gov.tr
doi:10.3906/sag-0912-454

An analysis of HER-2/neu gene status in invasive ductal carcinomas using immunohistochemistry and fluorescence in situ hybridization

Reşit Doğan KÖSEOĞLU[1], Ahmet MÜSLEHİDDİNOĞLU[2], Ünal ERKORKMAZ[3], Jülide Saliha CAFERLER[4], Pervin DÜRER[2], Yavuz Hakan ÖZÖN[4]

Aim: To determine the concordance rates between results from immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) assays for the HER-2/neu gene in cases of invasive ductal carcinoma. IHC and FISH are 2 of the most frequently used methods to determine HER-2/neu gene status in invasive breast carcinomas.

Materials and methods: The present study includes 59 female patients with invasive ductal carcinoma. The mean age was 57.8 years. Axillary lymph node status could be evaluated in 30 patients with axillary dissection. HER-2/neu gene overexpression and amplification were analyzed in primary tumor tissues by IHC and FISH assays, respectively. The evaluation of the IHC assay was performed using a score of 0, 1+, 2+, or 3+, while a HER-2/neu-to-CEP17 ratio greater than 2 was accepted as positive for HER-2/neu gene amplification.

Results: The overall rate of HER-2/neu gene amplification was 28.8% (17 cases) among all members of the study group, while this rate was 48% (12 cases) in the group with axillary lymph node metastasis. The rate of HER-2/neu overexpression (score of 3+) was 11.9% (7 cases). HER-2/neu gene amplification was associated with tumor grade ($P < 0.05$). There was a significant relationship between the results of the IHC and FISH assays ($P < 0.001$). All of the cases with a score of 3+ were FISH-positive, while 97% of cases with a score of 0 and 44% of cases with a score of 1+ were FISH-negative. HER-2/neu gene amplification was determined in 80% of the cases with a score of 2+. Among cases with positive lymph node status, the concordance rates were 100% in cases with scores of 0, 2+, and 3+, while this rate was found to be 29% in cases with a score of 1+.

Conclusion: Our found rate (11.9%) of HER-2/neu overexpression was lower than the rates reported in the literature, while our HER-2/neu gene amplification rate (28.8%) was compatible with the reported rates. HER-2/neu gene amplification was associated with tumor grade. Although high rates of concordance between HER-2/neu gene overexpression and amplification in cases with scores of 2+ and 3+ were obtained, the discordance rate in cases with a score of 1+ was higher than those of other studies. The discordance rate in cases with a score of 1+ and positive lymph node status was higher than that of the total study group. According to our results, a score of 1+ indicated less conclusive immunostaining for the HER-2/neu gene, and cases with scores of 1+ in the metastatic group had a higher rate of HER-2/neu gene amplification than that of the total study group. Our results show that FISH analysis for HER-2/neu gene status should be performed in cases with an immunostaining score of 1+, especially in those cases with positive axillary lymph node results.

Key words: Invasive breast carcinoma, HER-2/neu, FISH, IHC

Received: 15.12.2009 – Accepted: 11.10.2010

[1] Department of Pathology, Faculty of Medicine, Gaziosmanpaşa University, Tokat - TURKEY
[2] Department of Pathology, Tokat Cevdet Aykan State Hospital, Tokat - TURKEY
[3] Department of Biostatistics, Faculty of Medicine, Gaziosmanpaşa University, Tokat - TURKEY
[4] Genetics Health Services, İstanbul - TURKEY
Correspondence: *Reşit Doğan KÖSEOĞLU, Department of Pathology, Faculty of Medicine, Gaziosmanpaşa University, 60100 Tokat - TURKEY*
E-mail: residdogan@hotmail.com

İnvaziv duktal karsinomalarda immünohistokimya ve floresan in situ hibridizasyonu ile HER-2/neu gen analizi

Amaç: İnvaziv meme karsinomalarında HER-2/neu gen durumunu belirlemek için immünohistokimya (İHK) ve fluoresan in situ hibridizasyon (FİSH) en sık olarak kullanılan 2 metotdur. Çalışmamızdaki amacımız invaziv duktal karsinoma olgularımızda HER-2/neu geni için İHK ve FİSH ile elde edilen sonuçlar arasındaki uyumu analiz etmekti.

Yöntem ve gereç: Çalışmaya invaziv duktal karsinoma tanısı konmuş 59 kadın hasta dahil edildi. Ortalama yaş 57,8 idi. Aksiller lenf düğümü durumu, aksiller disseksiyon uygulanmış 30 hastada değerlendirilebildi. HER-2/neu gen ekspresyonu ve amplifikasyonu primer tümör dokularında İHK ve FİSH metotları ile analiz edildi. İHK analizini yorumlamak için skor 0, 1+, 2+ ve 3+ şeklinde skorlama uygulanırken HER-2/neu : CEP17 oranı 2 ve üzerinde olan olgular HER-2/neu gene amplifikasyonu için pozitif kabul edildi.

Bulgular: Çalışmamızda olguların tamamı dikkate alındığında HER-2/neu gen amplifikasyon oranı % 28,8 (17 olgu) iken bu oran lenf düğümü pozitif grupta % 48 (12 olgu) idi. Serimizde HER-2/neu aşırı ekspresyon (skor 3+) oranı % 11,9 olarak saptandı (7 olgu). HER-2/neu gen amplifikasyonu tümör derecesi ile ilişkiliydi ($P < 0,05$). İHK ve FİSH sonuçları arasındaki ilişki anlamlıydı ($P < 0,001$). Skore 3+ olan olguların tümü FİSH pozitif iken skor 0 olanların % 97'si, skor 1+ olanların % 44'ü FİSH negatif idi. Skore 2+ olguların % 80' inde HER-2/neu gen amplifikasyonu saptandı. Lenf düğümü pozitif skor 0, 2+ ve 3+ olgularda İHK ve FİSH sonuçları arasındaki uyum oranı % 100 iken skor 1+ olgularda bu oran % 29 idi.

Sonuç: Sonuç olarak, kendi serimizdeki HER-2/neu aşırı ekspresyon oranı (% 11,9) literatürde rapor edilmiş oranlardan düşük iken HER-2/neu gen amplifikasyon oranı (% 28,8) literatür ile uyumluydu. Çalışmamızda HER-2/neu gen amplifikasyonu tümör derecesi ile ilişkiliydi. Skor 2+ ve 3+ olgularda HER-2/neu aşırı ekspresyon ve gen amplifikasyon sonuçları arasındaki uyum oranı yüksek iken skor 1+ olgulardaki uyumsuzluk oranı diğer rapor edilmiş oranlara göre daha yüksekti. Pozitif lenf düğümlü skor 1+ olgulardaki uyumsuzluk oranı total çalışma grubununkinden daha yüksekti. Sonuçlarımıza göre, HER-2/neu için skor 1+ immünboyanma daha belirsiz bir sonuçtu ve metastatik hasta grubunda skor 1+ olgular çalışma grubunun tamamından daha yüksek HER-2/neu gen amplifikasyon oranına sahipti. Sonuçlarımız, özellikle aksiller lenf düğümü pozitif olan skor 1+ olgularda HER-2/neu gen durumunun FİSH analizi ile değerlendirilmesi gerektiğine işaret etmektedir.

Anahtar sözcükler: İnvaziv meme karsinomu, HER-2/neu, FİSH, İHK

Introduction

The oncogene HER-2/neu (c-erbB-2) is a protooncogene located on chromosome 17q that encodes a transmembrane tyrosine kinase growth factor receptor that belongs to the epidermal growth factor receptor (EGFR) or HER family (1,2). Overexpression or amplification of HER-2/neu has been reported in approximately 10%-30% of invasive breast carcinomas (3). In particular, invasive ductal carcinomas show a higher frequency of HER-2/neu gene amplification than other histological types of invasive breast carcinomas (4,5). Amplification of the HER-2/neu gene causes amplified transcriptive activity, tumorigenesis, and tumor metastasis (2). Many studies have shown that HER-2/neu overexpression or amplification is related to increased disease recurrence, metastasis, and shortened survival (6-9). According to the results of many studies, high proliferative index, high histological grade, lack of estrogen and progesterone receptors, p53 accumulation, and positive axillary lymph nodes were clinicopathological parameters related to HER-2/neu gene overexpression or amplification (1,2,4,10-12). In addition to the prognostic value of HER-2/neu, it is also important from the standpoint of therapeutic strategy. HER-2/neu represents an ideal therapeutic target because it is accessible as a cell surface receptor and is expressed at high levels in invasive breast cancers (2). A monoclonal antibody, trastuzumab (Herceptin), has been shown to be effective in patients with metastatic disease who are strongly positive for HER-2/neu and fail to respond to treatment with chemotherapy (1,13). Moreover, chemotherapy combined with trastuzumab has been shown to be more effective than chemotherapy alone (14). However, trastuzumab treatment is potentially toxic and has a high cost. For these reasons, the accurate testing of HER-2/neu should be

ensured by appropriate methods in order to prevent the unnecessary administration of trastuzumab treatment in patients with no amplification of HER-2/neu (2).

Various methods are used to determine HER-2/neu gene status in tumor tissues. Immunohistochemistry (IHC) is the most frequently used method for assessing gene overexpression. Fluorescence in situ hybridization (FISH) is a more recent technique that determines gene amplification in tumor tissues. Both techniques are applied to formalin-fixed and paraffin-embedded tissues. IHC evaluation of HER-2/neu status is a simpler and more practical method that can be performed at a low cost in all pathology laboratories. However, IHC testing can present some problems caused by different antibodies, tissue processing, and various interpretations of results. FISH is a highly accurate method with excellent sensitivity and specificity for the detection of HER-2/neu gene amplification. FISH has a low interlaboratory variability thanks to a standardized threshold that is used to determine HER-2/neu gene amplification (9,10,15). The determination of HER-2/neu gene amplification using FISH is considered to be the best indicator for beginning trastuzumab treatment in patients with invasive breast carcinoma (16). FISH is costly and more technically difficult, however, and for these reasons this technique cannot be utilized in many pathology laboratories (1).

Our primary aim in this study was to compare the values of expression and amplification of the HER-2/neu gene as analyzed by IHC and FISH. In addition, we analyzed whether or not there was any relationship between HER-2/neu gene status and clinicopathological parameters such as tumor grade, tumor size, and axillary metastasis status.

Materials and methods

Fifty-nine female patients with invasive ductal carcinoma were included in this study. Of these, 31 cases were obtained from the archive of Gaziosmanpaşa University's Department of Pathology while 28 cases were obtained from the Department of Pathology of Tokat State Hospital. Modified radical mastectomy and axillary dissection procedures had been performed in 30 cases, while 29 cases had undergone excisional biopsy without axillary dissection. Clinicopathological data such as age, tumor size, and axillary lymph node status were obtained from pathology reports and patients' medical files. The ages of the patients were not categorized for statistical analysis and were analyzed as a continuous variable. Tumor size was categorized as ≤20 mm, 21-39 mm, and ≥40 mm. The numbers of dissected lymph nodes in the axillary dissections of 30 cases were between 6 and 52. After the paraffin blocks and slides of the cases were obtained, the slides were reviewed and the diagnoses were confirmed. All cases were graded according to the histological grading scheme of the Bloom-Richardson system. Information on tumor size and axillary metastasis status was noted from the pathology reports of each case. IHC assays for the HER-2/neu gene were performed in the Department of Pathology at Gaziosmanpaşa University, while FISH assays were performed in the laboratories of Genetics Health Services, İstanbul. The analyses of both IHC and FISH were performed on the tissues of primary breast carcinoma.

Immunohistochemistry

In each case, tumor tissue fragments extracted from different areas of the tumors by punch extractor were prepared as a single paraffin block. These paraffin-embedded tissues were cut to a thickness of 4 μm and the sections were deparaffinized and rehydrated. Antigen retrieval was performed by boiling the sections in 0.01 M sodium citrate buffer, pH 6, for 10 min in a microwave oven. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide and nonspecific antibody binding was inhibited using normal goat serum. Sequentially, the sections were incubated with c-erbB-2/HER-2/neu Ab-17 antibody (clone, e2-4001+3B5, 1:400; Thermo Fisher Scientific, Fremont, CA, USA) for 30 min. Biotinylated goat antirabbit IgG and streptavidin-biotin peroxidase complexes were then used. Immunoreactivity was illustrated using aminoethylcarbazole and the slides were counterstained with Mayer's hematoxylin.

The staining results were scored as 0 (no staining observed, or membrane staining in <10% of the tumor cells), 1+ (faint or barely perceptible focal membrane staining in >10% of tumor cells), 2+ (weak to moderate staining of the complete cell membrane

in >10% of the tumor cells) (Figure 1A), or 3+ (strong staining of the complete membrane in >10% of the tumor cells) (Figure 1B). Scores of 0 and 1+ were considered negative for HER-2/neu expression, scores of 3+ were considered positive, and scores of 2+ were accepted as weak/borderline positives or inconclusive results. Positive and negative controls were used to validate each run of the assay.

Fluorescence in situ hybridization

The same paraffin blocks used in IHC assays were also used in FISH assays. FISH, in 2 colors, was carried out on tissue sections 4 μm thick. After the tissue sections were deparaffinized, the sections were treated with 0.2 N HCl for 20 min and then washed in distilled water for 2 min. The slides were incubated in a pretreatment solution at 80 °C for 30 min and washed in distilled water for 2 min. The slides were then immersed in a protease solution at 37 °C for 30 min and rinsed with distilled water for 2 min. Next, the slides were fixed in a postfixative solution for 2 min at room temperature. After the sections were washed in distilled water, a serial dehydration procedure was performed in 70%, 85%, and 100% ethanol at 45-50 °C. For hybridization, 10 μL of the PathVysion HER-2 DNA Probe Kit (Probes, Vysis LSI HER-2/neu and Vysis CEP17; Abbott Laboratories, Abbott Park, IL, USA) was applied to the slides. After overnight hybridization at 37 °C in a hybridization device, the slides were washed with a posthybridization wash buffer at 72 °C for 2 min. The tissue sections were counterstained with 10 μL of 4,6-diamino-2-phenylindole.

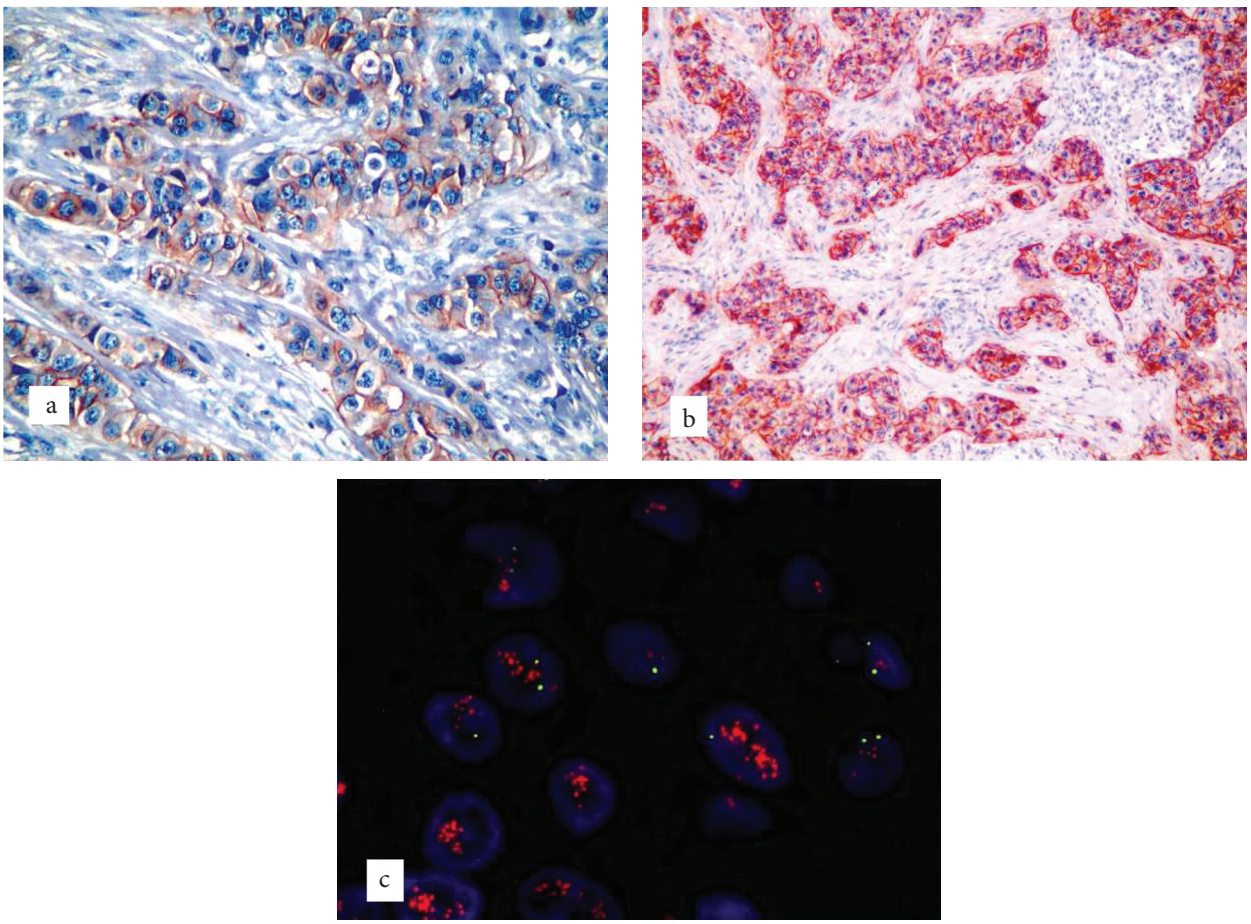

Figure 1. A) Weak, moderately complete membranous HER-2/neu immunostaining (score of 2+) in invasive ductal carcinoma cells (AEC, ×30); B) strong, complete membranous immunostaining (score of 3+) with HER-2/neu antibody in more than 10% of invasive ductal carcinoma cells (AEC, ×10); C) invasive ductal carcinoma cells showing HER-2/neu amplification by FISH assay.

Each tissue section was scanned initially at low power to assess heterogeneity and to identify appropriate areas of tumor tissue with clearly defined, nonoverlapping nuclei. The ×60 objective was then used to score signals in 60 nonoverlapping tumor cell nuclei in order to determine the average number of HER-2/neu and CEP17 copies per cell for each tissue specimen. The ratio of these averages was used to determine the presence of HER-2/neu gene amplification. Specimens with an HER-2/neu-to-CEP17 ratio greater than 2 were scored as positive for HER-2/neu gene amplification (Figure 1C).

Data analysis

Each of the parameters, such as age, nuclear grade, tumor size, axillary lymph node status, and the staining results of c-erbB2/HER-2/neu analysis, was compared with the others in this study. The chi-square test was used to compare categorical variables, while continuous variables were compared using 1-way ANOVA and the independent 2-sample t-test. Cramer's coefficient was used for the evaluation of concordance among variables and $P < 0.05$ was accepted as significant.

Results

Patients' ages ranged from 35 to 88 years. The mean age was 57.8 years. The sizes of tumors were between 10 and 100 mm, and 2 cases were excluded from analysis because the sizes of the tumors could not been determined from pathology reports. Histological grade 1 accounted for 9 cases (15.3%), while grades 2 and 3 accounted for 39 cases (66.1%) and 11 cases (18.6%), respectively. Lymph node metastasis was found in 25 (83.3%) out of 30 cases in which mastectomy and axillary dissection had been performed. The other 5 cases were free of axillary lymph node metastasis. Immunohistochemical HER-2/neu expression was not seen in 38 cases (64.4%). The immunostaining score of 7 cases (11.8%) was 3+, while scores of 1 + and 2+ were determined in 9 (15.3%) and 5 (8.5%) of the cases, respectively. When analyzed using FISH assay, 17 cases (28.8%) were determined to show HER-2/neu gene amplification (Table 1).

Age and tumor size were not related to HER-2/neu status ($P > 0.05$). The relationship between tumor grade and HER-2/neu expression was not significant. All cases of grade 1 were negative for HER-2/neu expression (7 cases, score of 0; 2 cases, score of 1+). Most grade 2 tumors were also found to be negative for HER-2/neu expression. In grade 2 tumors, 24 cases (61.5%) had a score of 0, while only 4 cases (10.3%) had scores of 3+. The rate of cases with a score of 3+ among grade 3 tumors (3 cases, 27.2%) was higher than those of grade 1 and 2 cases without a statistically significant relationship ($x^2 = 8.826$, $P = 0.184$) (Table 1).

A significant relationship was observed between tumor grade and HER-2/neu gene amplification. All 9 of the cases with grade 1 tumors were negative for HER-2/neu gene amplification. However, 33.3% (13 cases) of the cases with grade 2 tumors and 36.4% (4 cases) of the cases with grade 3 tumors showed HER-2/neu gene amplification. No cases with grade 1 tumors demonstrated HER-2/neu gene amplification. Most of the HER-2/neu-amplified tumors (13 cases, 76.5%) were grade 2, while only 4 cases (23.5%) with HER-2/neu amplification were grade 3. While 61.9% of the cases without HER-2/neu gene amplification were grade 2, only 7 cases (16.7%) without HER-2/neu gene amplification were grade 3. Tumor grade was therefore associated with HER-2/neu gene amplification ($x^2 = 6.787$, $P = 0.034$) (Table 1).

The relationship between the results of IHC and FISH assays performed for HER-2/neu gene status was statistically significant ($x^2 = 44.238$, $P < 0.001$). This can be seen by the fact that 7 (41.2%) of the FISH-positive cases (i.e. cases with HER-2/neu gene amplification) had a score of 3+ for HER-2/neu expression. The rate of FISH-positive cases with an immunostaining score of 2+ was 23.5%. Only 1 case (5.9%) with HER-2/neu gene amplification did not show any immunohistochemical staining for HER-2/neu. The majority (88.1%) of FISH-negative cases (i.e. cases without HER-2/neu gene amplification) were found to have a score of 0 for HER-2/neu immunostaining. Immunostaining scores of 3+ for HER-2/neu were not determined in the FISH-negative group; only 1 FISH-negative case (2.4%) had a score of 2+, while 4 cases (9.5%) had scores of 1+. All of the cases (100%) with immunostaining scores of 3+ and 80% of cases with scores of 2+ were FISH-positive. The majority (37 cases, 97.4%) of

Table 1. Association between HER-2/neu gene status and other pathological characteristics.

	Her-2/neu expression n (%)						**Her-2/neu gene amplification** n (%)			
Grade	**0**	**1+**	**2+**	**3+**	**Total**	χ^2 **and P values**	**FISH (+)**	**FISH (-)**	**Total**	χ^2 **and P values**
1	7 (77.8)	2 (22.2)	0	0	9 (15.3)	$\chi^2 = 8.826$ P = 0.184	0	9 (100)	9 (15.3)	$\chi 2 = 6.787$ P = 0.034
2	24 (61.5)	6 (15.4)	5 (12.8)	4 (10.3)	39 (66.1)		13 (33.3)	26 (66.7)	39 (66.1)	
3	7 (63.6)	1 (9.1)	0	3 (27.3)	11 (18.6)		4 (36.4)	7 (63.6)	11 (18.6)	
Total	**38 (64.4)**	**9 (15.2)**	**5 (8.5)**	**7 (11.9)**	**59 (100)**		**17 (28.8)**	**42 (71.2)**	**59 (100)**	
Size (mm)										
≤20	10 (76.9)	2 (15.4)	0	1 (7.7)	13 (22.8)	$\chi^2 = 9.076$ P = 0.430	1 (7.7)	12 (92.3)	13 (22.8)	$\chi^2 = 7.159$ P = 0.067
21 -39	19 (54.3)	7 (20)	4 (11.4)	5 (14.3)	35 (61.4)		14 (40)	21 (60)	35 (61.4)	
≥40	7 (77.8)	0	1 (11.1)	1 (11.1)	9 (15.8)		2 (22.2)	7 (77.8)	9 (15.8)	
Total	**36 (63.1)**	**9 (15.8)**	**5 (8.8)**	**7 (12.3)**	**57 (100)**		**17 (100)**	**40 (100)**	**57 (100)**	
Metastasis										
Positive	11 (44)	7 (28)	3 (12)	4 (16)	25 (83.3)	$\chi^2 = 1.112$ P = 0.774	12 (48)	13 (52)	25 (83.3)	$\chi^2 = 1.433$ P = 0.355
Negative	1 (20)	2 (40)	1 (20)	1 (20)	5 (16.7)		1 (20)	4 (80)	5 (16.7)	
Total	**12 (40)**	**9 (30)**	**4 (13.3)**	**5 (16.7)**	**30 (100)**		**13 (43.3)**	**17 (56.7)**	**30 (100)**	

the cases with immunostaining scores of 1+ were FISH-negative. Only 1 (2.6%) case showed HER-2/neu gene amplification. Among the cases with immunostaining scores of 1+, 5 cases (55.6%) were FISH-positive while 4 cases (44.4%) did not show HER-2/neu gene amplification (Table 2).

In this study, only 30 out of 59 cases underwent axillary dissection and we therefore had knowledge about the axillary lymph node status of only these 30 cases. As a distinct group, these 30 cases were statistically evaluated for HER-2/neu gene amplification and expression according to lymph node status (Table 1). The findings of this evaluation showed that 11 (44%) of 25 cases with axillary lymph node metastasis did not show any immunostaining for HER-2/neu. A total of 14 cases (56%) showed HER-2/neu expression; 28% (7 cases) of these had a score of 1+, while 3 cases (12%) were found to have a score of 2+ and 4 cases (16%) had a score of 3+ for HER-2/neu expression. The distribution of the 5 cases without metastasis was about equal with regard to the categories of scores for HER-2/neu expression. A majority of the cases in all categories of HER-2/neu immunostaining scores showed lymph node metastasis. A significant relationship between axillary lymph node status and HER-2/neu expression was not determined ($x^2 = 1.112$, P = 0.774). Similarly, HER-2/neu amplification was also not associated with axillary lymph node status ($x^2 = 1.433$, P = 0.355). In the group subject to axillary dissection, 12 (92.3%) of the cases with HER-2/neu gene amplification and 13 (76.5%) of the FISH-negative cases had axillary lymph node metastasis. While 12 of 25 cases (48%) with axillary lymph node metastasis were FISH-positive, this rate was 20% in the cases without axillary lymph node metastasis and the difference was not statistically significant.

A statistically significant association was found between the results of FISH and IHC assays in 25 cases with axillary lymph node metastasis (x^2 =

Table 2. Association between the results of FISH and IHC for HER-2/neu gene status.

Her-2/neu gene amplification	Her-2/neu expression n (%)				Total	χ^2 and P values
	0	1+	2+	3+		
FISH (+)	1 (5.9)	5 (29.4)	4 (23.5)	7 (41.2)	17 (28.8)	
FISH (-)	37 (88.1)	4 (9.5)	1 (2.4)	0	42 (71.2)	$\chi^2 = 44.238$ $P < 0.001$
Total	**38 (64.4)**	**9 (15.2)**	**5 (8.5)**	**7 (11.9)**	**59 (100)**	

26.242, P < 0.001). Meanwhile, 4 (33.3%) of 12 cases with HER-2/neu gene amplification had a score of 3+, while 3 cases (25%) had a score of 2+ for HER-2/neu expression. The remaining 5 cases had a score of 1+. There was no case with a score of 0 among the FISH-positive cases with axillary lymph node metastasis. None of the 13 FISH-negative cases with axillary lymph node metastasis showed immunostaining scores of 2+ or 3+ for HER-2/neu. Among the FISH-negative cases, 11 cases (84.6%) had a score of 0, while only 2 cases (15.4%) had a score of 1+ for HER-2/neu expression. All of the cases showing immunostaining scores of 2+ or 3+ for HER-2/neu were FISH-positive, while all cases with scores of 0 were FISH-negative. In the group with scores of 1+ for HER-2/neu immunostaining, 71.4% (5 cases) of the cases showed HER-2/neu gene amplification (Table 3).

Discussion

The analyses that are performed to detect HER-2/neu amplification and/or overexpression in cases of invasive breast carcinoma, especially invasive ductal carcinoma, have considerable importance in the determination of prognosis and sensitivity/resistance for chemotherapy and hormone therapy. Detection of HER-2/neu status also serves to select patients for treatment with a monoclonal antibody targeting the HER-2/neu protein. Because of the toxicity and high cost of this monoclonal antibody treatment, the accurate assessment of patients selected for this treatment is very important (1,2). HER-2/neu gene status can be determined by using different analysis methods, such as the Southern blot technique, the western blot, the dot blot, quantitative polymerase chain reaction, IHC, and FISH (17,18). Of these methods, the 2 most frequently employed are IHC

Table 3. Association between the results of FISH and IHC in cases with metastatic axillary lymph node.

Her-2/neu gene amplification	Her-2/neu expression n (%)				Total	χ^2 and P values
	0	1+	2+	3+		
FISH (+)	0	5 (41.7)	3 (25)	4 (33.3)	12 (48)	
FISH (-)	11 (84.6)	2 (15.4)	0	0	13 (52)	$\chi^2 = 26.242$ $P < 0.001$
Total	**11 (44)**	**7 (28)**	**3 (12)**	**4 (16)**	**25 (100)**	

and FISH. These methods are considered superior to other techniques due to the fact that amplification and overexpression of the gene is localized to tumor cells and it is possible to prevent the distortion of results arising from surrounding stromal cells. Another superior feature of both techniques is that they can be performed on archival material. In addition to these benefits, however, both techniques have some disadvantages and there may be discrepancies between IHC and FISH results. Factors that may possibly cause the discordance between the 2 techniques are usually related to IHC. These factors include variable fixation methods, tissue processing, immunostaining procedure, subjectivity in the grading of expression, interpretation at low levels of gene amplification, polysomy of chromosome 17, differences in the specificities/sensitivities of commercially available antibodies, and the identification of different c-erb-B-2 protein domains by these antibodies (2,19). The rates of concordance between IHC and FISH have varied in different studies (11,18-21). The consecutive scores have been defined in immunohistochemical evaluation of HER-2/neu. Scores of 0 and 1+ are considered to be a negative result for HER-2/neu expression, while a score of 3+ is accepted as a positive result. An immunostaining score of 2+ is defined as an inconclusive result. Although FISH analysis is especially suggested for scores of 2+, there are also authors that recommend the FISH assay as a supplement to all scores of immunostaining (19). Because using FISH as a screening test is expensive and time-consuming, many studies have advocated the combined approach of IHC as a primary screening modality followed by FISH assay for IHC-inconclusive cases. In this situation, the concordance between the results of IHC and FISH is of major importance. In the literature, this concordance rate was found to be higher (49%-100%) for cases with scores of 3+, while lower values (0%-42%) for scores of 2+ were reported (2,11,19,20,22-24). The concordance rate between FISH and IHC also had a high value (higher than 89%) in cases with negative results for HER-2/neu expression, i.e. cases with scores of 0 and 1+ (1,2,19-21). Data from the literature show that scores of 2+ are inconclusive for HER-2/neu status and that FISH analysis should be performed in order to determine HER-2/neu gene status in these cases. In our study, the concordance rates between FISH and IHC for scores of 3+ and 2+ were 100% and 80%, respectively, while this rate was 87.2% for cases with negative results. When compared with other studies, our results showed some differences, except for cases with scores of 3+. The concordance rate in cases with scores of 2+ was higher than those of other studies. However, this rate should be taken into consideration cautiously because there were only 5 cases in this group. The low number of cases with a score of 2+ was a limiting factor for interpretation. When the concordance between FISH and IHC in the cases with a score of 1+ was analyzed, however, the concordance rate (44.4%) in our study was noticeably lower than other rates reported in the literature. This rate was higher than 90% in the studies by Prati et al. (1) and McCormick (20). In our study, a low rate of concordance in cases with a score of 1+ indicates that these cases should be considered inconclusive for HER-2/neu status as determined by IHC assay. The concordance rate (87.2%) in cases with negative HER-2/neu expression was slightly lower than those (89%-96%) of other studies (1,2,19,20). In our study, the concordance rates between FISH and IHC in cases with positive and negative results for HER-2/neu expression were similar to the rates reported in the literature. On the other hand, the present study revealed that discordances were present between FISH and IHC in cases with scores of 2+ and 1+. The association between IHC and FISH was statistically meaningful in the present study (x^2 = 44.238, P < 0.001). All cases with scores of 3+ were positive for HER-2/neu gene amplification, and 87.2% of cases with negative results for HER-2/neu expression did not show HER-2/neu gene amplification.

We analyzed concordance between the results of IHC and FISH in the cases with axillary lymph node metastasis as a separate group. There was also a statistically significant association between the results of IHC and FISH in this group (x^2 = 26.242, P < 0.001). The concordance rates between results of IHC and FISH in groups with immunostaining scores of 0, 2+, and 3+ were 100%, while the concordance in the group with scores of 1+ was 28.6%. This rate was 72.2% in the cases with negative results for HER-2/neu expression. These latter 2 percentages, which refer only to the 30 cases in which patients underwent axillary dissection, were lower than the rates for the

entire group of 59 patients. Our study showed that in cases with axillary lymph node metastasis, a score of 1+ was to be viewed as an inconclusive result for HER-2/neu status analysis by IHC. Additionally, cases with scores of 1+ in the metastatic group showed a higher frequency (71.4%) of HER-2/neu gene amplification than that (55.6%) of cases with a score of 1+ in the analysis of all cases, including those with and without axillary dissection. The discordance rate (71.4%) between expression and amplification of HER-2/neu in cases with a score of 1+ in the metastatic group was higher than that (55.6%) of the total study group including all cases; cases with scores of 1+ and axillary lymph node metastasis showed a higher tendency to be FISH-positive for the HER-2/neu gene.

In our study, the rate of HER-2/neu gene amplification as determined by FISH assay was 28.8% (17 cases) in total from the entire study group, while this rate was 48% (12 cases) in the group with axillary lymph node metastasis. The rate of HER-2/neu overexpression (score of 3+) as determined by IHC assay was 11.9% (7 cases). Although this rate was lower than those reported in the literature (1,2,11,17-21), our rate of HER-2/neu gene amplification was compatible with those of other studies (1,2,4,11,17-21,25). The ranges of HER-2/neu overexpression and amplification rates according to the references cited in the present study were 14.6%-44.1% and 15%-39.1%, respectively. The low rate of HER-2/neu overexpression in the present study may have arisen from various technical aspects of the procedure, such as the immunohistochemical staining technique, interpretation and scoring of immunostaining results, or the antibody clone used. In fact, IHC analysis was repeated in the cases showing discordance with the results of the FISH analysis, and the IHC results were not changed. For this reason, factors other than the immunohistochemical staining technique itself became the primary focus of our attempts to explain the low rate of HER-2/neu overexpression. When the overall results of the analysis were considered, however, a concordance between the results of IHC and FISH was found to be present in this study.

Tumor grade was correlated with FISH-determined HER-2/neu gene status in a study by Prati et al (1). Poorly differentiated infiltrating ductal carcinomas had a higher frequency of HER-2/neu gene amplification than moderately and well-differentiated tumors (4). Many studies have showed strong correlations between tumor grade and HER-2/neu status as determined by either IHC alone (12,26-28) or by both IHC and FISH (29). In the present study, the tumors of cases with immunostaining scores of 2+ and 3+ were grades 2 or 3. Conversely, cases found to be negative for HER-2/neu expression were also mostly grade 2 tumors. Our study failed to show a significant association between tumor grade and HER-2/neu expression (x^2 = 8.826, P = 0.184). Although HER-2/neu amplified and nonamplified tumors were also mostly grade 2, none of the cases with HER-2/neu gene amplification presented a grade 1 tumor. Grade 3 tumors made up 23.5% of the cases in the group of HER-2/neu amplified tumors; this rate was 16.7% in the FISH-negative group. The rate of grade 1 tumors in the FISH-negative group was 21.4%. A significant association was found between HER-2/neu gene amplification and tumor grade (x^2 = 6.787, P = 0.034).

The present study did not reveal an association among age, tumor size, or HER-2/neu expression and amplification. Very limited data concerning associations among age, size, and HER-2/neu status are available in the literature. In studies by Lee et al. (30) and Gong et al. (25), HER-2/neu overexpression was shown to be associated with larger tumor size, while a relationship was not determined between age and HER-2/neu overexpression. Ariga et al. (4) reported that HER-2/neu gene amplification did not show a significant relationship with age or tumor size.

Our study included 30 cases with axillary dissection; in these 30 cases, axillary metastasis status was compared with HER-2/neu status. Although the rate of HER-2/neu gene amplification in cases with positive axillary lymph node status (48%) was higher than that of nonmetastatic cases, HER-2/neu gene amplification was not related with axillary lymph node status in our study. Similarly, an association between HER-2/neu expression and axillary lymph node status was not also determined in the present study. Although a meaningful relationship between axillary lymph node metastasis and HER-2/neu status has been suggested (10,25), there have also been other studies (1,27,31,32), as well as our own findings, that did not show a significant association.

In conclusion, the present study showed a lower HER-2/neu overexpression rate than those of other studies in the literature, while our HER-2/neu gene amplification rate was compatible with other reported rates. A significant association between HER-2/neu gene amplification and histological grade was observed. According to our results, an immunostaining score of 1+ was a less conclusive result for HER-2/neu status as determined by IHC, and cases with scores of 1+ in the metastatic group had a higher rate of HER-2/neu amplification. Our results show that FISH analysis for HER-2/neu gene status should be performed in the cases with scores of 1+, particularly those that have a positive axillary lymph node status.

References

1. Prati R, Apple SK, He J, Gornbein JA, Chang HR. Histopathologic characteristics predicting HER-2/neu amplification in breast cancer. Breast J 2005; 11: 433-9.
2. Pothos A, Plastira K, Plastiras A, Vlachodimitropoulos D, Goutas N, Angelopoulou R. Comparison of chromogenic in situ hybridisation with fluorescence in situ hybridisation and immunohistochemistry for the assessment of her-2/neu oncogene in archival material of breast carcinoma. Acta Histochem Cytochem 2008; 41: 59-64.
3. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE et al. Studies of the HER-2 neu proto-oncogene in human breast and ovarian cancer. Science 1989; 244: 707-12.
4. Ariga R, Zarif A, Korasick J, Reddy V, Siziopikou K, Gattuso P. Correlation of her-2/neu gene amplification with other prognostic and predictive factors in female breast carcinoma. Breast J 2005; 11: 278-80.
5. Hoff ER, Tubbs RR, Myles JL, Procop GW. Her2/neu amplification in breast cancer. Stratification by tumor type and grade. Am J Clin Pathol 2002; 117: 916-21.
6. Ross JS, Fleisher JA. HER-2/neu (c-erb-B2) gene and protein in breast cancer. Am J Clin Pathol 1999; 112: 53-67.
7. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 1987; 235: 177-82.
8. Slamon DJ. Biologic approaches to managing advanced breast cancer. Cancer Control 1999; 6: 7-11.
9. Ross JS, Fletcher JA, Bloom KJ, Linette GP, Stec J, Symmans WF et al. Targeted therapy in breast cancer: the HER-2/neu gene and protein. Mol Cell Proteomics 2004; 3: 379-98.
10. Kaptain S, Tan L, Chen B. Her-2/neu and breast cancer. Diagn Mol Pathol 2001; 10: 139-52.
11. Ridolfi RL, Jamehdor MR, Arber JM. HER-2/neu testing in breast carcinoma: a combined immunohistochemical and fluorescence in situ aproach. Mod Pathol 2000; 13: 866-73.
12. Harłozińska A, Bar JK, Wenderski R, Bebenek M. Relationship between c-erbB-2 oncoprotein, epidermal growth factor receptor, and estrogen receptor expression in patients with ductal breast carcinoma. Association with tumor phenotypes. In Vivo 1996; 10: 217-22.
13. Cobleigh MA, Vogel CL, Tripathy D, Robert NJ, Scholl S, Fehrenbacher L et al. Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. J Clin Oncol 1999; 17: 2639-2648.
14. Slamon D, Leyland-Jones B, Shak S, Paton V, Bajamonde A, Fleming T et al. Addition of Herceptin (humanized anti-HER2 antibody) to first line chemotherapy for HER2 overexpressing metastatic breast cancer (HER2+/MBC) markedly increases anticancer activity: a randomized multinational controlled phase III trial. Proc Am Soc Clin Oncol 1998; 17: 98a.
15. Bartlett JM, Going JJ, Mallon EA, Watters AD, Reeves JR, Stanton P et al. Evaluating HER2 amplification and overexpression in breast cancer. J Pathol 2001; 195: 422-28.
16. Vogel CL, Franco SX. Clinical experience with trastuzumab (Herceptin). Breast J 2003; 9: 452-62.
17. Onody P, Bertrand F, Muzeau F, Bièche I, Lidereau R. Fluorescence in situ hybridization and immunohistochemical assays for HER-2/neu status determination: application to node-negative breast cancer. Arch Pathol Lab Med. 2001; 125: 746-50.
18. Jacobs TW, Gown AM, Yaziji H, Barnes MJ, Schnitt SJ. Comparison of fluorescence in situ hybridization and immunohistochemistry for the evaluation of HER-2/neu in breast cancer. J Clin Oncol. 1999; 17: 1974-82.
19. Hammock L, Lewis M, Phillips C, Cohen C. Strong HER-2/neu protein overexpression by immunohistochemistry often does not predict oncogene amplification by fluorescence in situ hybridization. Hum Pathol 2003; 34: 1043-7.
20. McCormick SR, Lillemoe TJ, Beneke J, Schrauth J, Reinartz J. HER2 assessment by immunohistochemical analysis and fluorescence in situ hybridization: comparison of HercepTest and PathVysion commercial assays. Am J Clin Pathol 2002; 117: 935-43.
21. Wang S, Saboorian MH, Frenkel EP, Haley BB, Siddiqui MT, Gokaslan S et al. Assessment of HER-2/neu status in breast cancer. Automated Cellular Imaging System (ACIS)-assisted quantitation of immunohistochemical assay achieves high accuracy in comparison with fluorescence in situ hybridization assay as the standard. Am J Clin Pathol 2000; 116: 495-503.

22. Lebeau A, Deimling D, Kaltz C, Sendelhofert A, Iff A, Luthardt B et al. HER-2/*neu* analysis in archival tissue samples of human breast cancer: Comparison of immunohistochemistry and fluorescence in situ hybridization. J Clin Oncol 2001; 19: 354-363.

23. Tubbs RR, Pettay JD, Roche PC, Stoler MH, Jenkins RB, Grogan TM. 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.

24. Perez EA, Roche PC, Jenkins RB, Reynolds CA, Halling KC, Ingle JN et al. HER-2 testing in patients with breast cancer: Poor correlation between weak positivity by immunohistochemistry and gene amplification by fluorescence in situ hybridization. Mayo Clin Proc 2002; 77: 148-54.

25. Gong Y, Booser DJ, Sneige N. Comparison of HER-2 status determined by fluorescence in situ hybridization in primary and metastatic breast carcinoma. Cancer 2005; 103: 1763-9.

26. van de Vijver MJ. Assessment of the need and appropriate method for testing for the human epidermal growth factor receptor-2 (HER-2). Eur J Cancer 2001; 37: 11-7.

27. Taucher S, Rudas M, Mader RM, Gnant M, Dubsky P, Bachleitner T et al. Do we need HER-2/neu testing for all patients with primary breast carcinoma? Cancer 2003; 98: 2547-53.

28. Tsuda H, Hirohashi S, Shimosato Y, Hirota T, Tsugane S, Watanabe S et al. Correlation between histologic grade of malignancy and copy number of c-erbB-2 gene in breast carcinoma. Cancer 1990; 8: 1794-800.

29. Sauer T, Wiedswang G, Boudjema G, Christensen H, Karesen R. Assessment of HER-2/neu overexpression and/or gene amplification in breast carcinomas: should in situ hybridization be the method of choice? APMIS 2003; 111: 444-50.

30. Lee A, Park WC, Yim HW, Lee MA, Park G, Lee KY. Expression of c-erbB2, cyclin D1 and estrogen receptor and their clinical implications in the invasive ductal carcinoma of the breast. Jpn J Clin Oncol. 2007; 37: 708-14.

31. Bilous M, Ades C, Armes J, Bishop J, Brown R, Cooke B et al. Predicting the HER2 status of breast cancer from basic histopathology data: an analysis of 1500 breast cancers as part of the HER2000 international study. Breast 2003; 12: 92-8.

32. Berger MS, Locher GW, Saurer S, Gullick WJ, Waterfield MD, Groner B et al. Correlation of c-erbB-2 gene amplification and protein expression in human breast carcinoma with nodal status and nuclear grading. Cancer Res 1988; 48: 1238-43.