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ARTICLE

Clinical Findings and HER-2/*neu* Gene Amplification Status of Breast Carcinoma Patients

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The study group was derived from the archival materials of 48 invasive intraductal breast cancer patients who had undergone partial mastectomy/axillary dissection. All patients included in the study had clinically $T_{1-2}N_0M_0$ invasive ductal carcinoma. To detect HER-2/*neu* status, fluorescent *in situ* hybridization was performed using a HER-2/*neu* locus-specific probe. Signals were counted and patients were classified in three groups according to signal ratios: signal ratio <2, group 1 (n=31); signal ratio 2-4, group 2 (n=11); signal ratio >4, group 3 (n=6). Ratios of axillary metastatic lymph nodes to dissected total lymph nodes were 17%, 23% and 83% in groups 1, 2 and 3 respectively (P=0.003). The number of metastatic axillary lymph nodes, and the ratio of microscopic metastatic lymph nodes were highest in group 3 (P=0.001 and P=0.008, respectively). No significant difference was observed between groups for distant metastasis in a 5-year follow-up period. Signal ratios decreased with estrogen receptor expression (P=0.03). Histopathologically, an irregular growth pattern of the tumor was observed in 100% of the patients in group 3, and in 54% and 60% in groups 1 and 2, respectively (P=0.04). Lymphovascular invasion of the tumor was significantly higher in group 3 compared to the other two groups (P=0.01). The extensive intraductal component ratio was the highest in group 3 (P=0.04). The appearance of desmoplastic reaction and lymphocyte infiltration did not show significant difference between the groups. Our results show that HER-2/*neu* signal ratio increases with lymphovascular invasion, an extensive intraductal component, irregular growth pattern and axillary metastasis in clinically $T_{1-2}N_0M_0$ invasive ductal carcinoma of the breast. (Pathology Oncology Research Vol 12, No 4, 211–215)

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

Introduction

The assessment of HER-2/*neu* gene amplification in breast cancer diagnosis and biology has become important in planning therapy. However, the testing time and for whom testing should be performed, as well as the method(s) to use are still controversial. Amplification of the HER-2/*neu* gene occurs in 25-30% of invasive ductal breast carcinoma.[1] Recently, the potential role of HER-2/*neu* alteration as a predictor of response or resistance to various therapeutic regimens in patients has become the focus of interest.[1] From the molecular point of view, amplification and enhanced expression of the HER-2/*neu* oncogene is often associated with reduced disease-free and overall survival in lymph node positive patients.[2,3]

The human HER-2 (HER-2/*neu*) gene is located on chromosome 17q and encodes a type I tyrosine kinase membrane receptor that has homology to epidermal growth factor.[4] HER-2 protein also plays an important role in normal cellular proliferation, is expressed at low levels in a variety of epithelial cell types including ductal epithelium of the breast. HER-2 protein overexpression most commonly results from gene amplification.[5] HER-2 induces cell division and stimulates factors facilitating cell motility and tumor metastasis.[4] When there is amplifica-

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tion of the gene, receptors are not degraded, instead they return to the cell surface. Therapeutic agents such as trastuzumab induce endocytosis and degradation of HER-2, and inhibit tumor cell growth.[4]

In this study, we examined the HER-2/*neu* status of tissue samples by fluorescent *in situ* hybridization, and attempted to demonstrate any correlation between clinical findings and HER-2/*neu* amplification.

Materials and Methods

Patients

Paraffin block sections from 48 patients on whom partial mastectomy/axillary dissection (PMAD) was performed were included in the study. All patients included in the study had clinical $T_{1-2}N_0M_0$ invasive ductal carcinoma and underwent radiotherapy after PMAD. For the evaluation of distant metastasis, all patients included in the study underwent preoperative chest x-ray for the assessment of pulmonary metastasis, abdominal ultrasonographic examination for the assessment of possible intraabdominal metastasis and Tc99-m scintigraphic examination for the assessment of bone metastasis. Diagnosis of invasive ductal carcinoma was proven by either core needle biopsy or open surgical biopsy prior to definitive surgical therapy. Patients with pure ductal carcinoma in situ (DCIS) were excluded from the study. All patients received adjuvant radiotherapy since our treatment approach was breast preserving surgical therapy (PMAD). All patients with tumor size of >1 cm and axillary metastasis received adjuvant chemotherapy. Axillary metastatic status, lymphovascular invasion (LVI), extensive intraductal component (EIC), irregular growth pattern (IGP), desmoplastic reaction (DR), and hypercellularity (HC) were assessed histopathologically. During the follow-up period, for assessment of distant metastasis, physical examination, abdominal ultrasonography, mammography and breast ultrasonography were performed.

FISH

3 to 4 mm thick tissue sections from surgical specimens were placed on poly-L-lysine coated slides and deparaffinized according to a slight modification of a procedure described previously.[6,7] After deparaffinization at 56 °C overnight, slides were dewaxed in xylene and treated with ethanol to eliminate xylene. Following pepsin digestion and fixation of slides, denaturation and hybridization were carried out in the HyBrite denaturation/hybridization system for FISH (Vysis, Downers Grove, IL, USA). Slides were denatured in 70% formamide, 2 × SSC at 75 °C for 3-5 min and the probe [Chromosome 17q12 (HER-2/*neu*)/alphasatellite 17 cocktail, dual color, direct labeled (Oncor, Qbiogene, UK)] was denatured at 96 °C for 5 min. Hybridization was carried out at 37 °C for 14-16 h. The slides were then washed in posthybridization wash buffer at 65 °C for 5 min and counterstained with DAPI.

Signals were counted in at least 200 cells for both HER-2/*neu* gene and chromosome 17 centromere signals under oil immersion at × 1000 magnification using the recommended filters. Results were expressed as the ratio of HER-2/*neu* signal (orange) to centromere 17 signal (green) and the patients were classified in to three groups: ratio <2, group 1 (n=31); ratio 2-4, group 2 (n=11); and ratio >4, group 3 (n=6). The expected ratio is 1 (no gene amplification). Thus, there is no amplification of the HER-2/*neu* gene in the first group of patients, while there is an amplification in patients with a ratio of HER-2/*neu* signal to centromere signal equal to or larger than 2.

As a control for probe hybridization and signal counts, five different tissue sections were hybridized with the HER-2/*neu* probe and the cut-off value was calculated as 2.27%.

Statistical analysis

The clinical features of the patients and correlations with signal ratios were analyzed by the chi-squared test; $P<0.05$ values were accepted as significant. The results were expressed as mean±SEM.

Results

The mean ages of the patients were 61 years (range, 52-81 years), 51 years (range, 44-70 years), and 49 years (range, 28-51 years) for group 1, 2 and 3, respectively; there was no statistically significant difference between the three groups ($P>0.05$).

In 31 patients the signal ratio was <2 (group 1), in 11 patients it was 2-4 (group 2), and in 6 patients the signal ratio was >4 (group 3) (*Figure 1*). Mean tumor size measured in formalin-fixed tissue and mean tumor-free margin

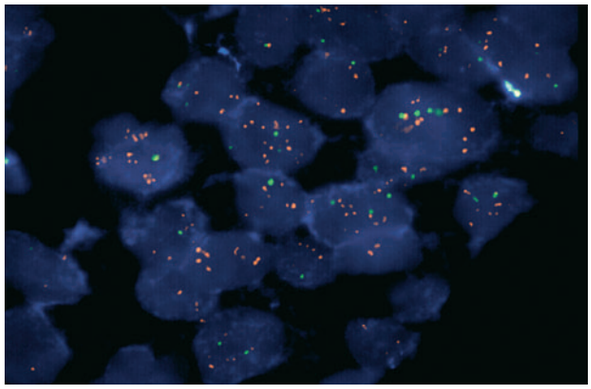

Figure 1. *HER-2/neu amplification in one of the patients in group 3. HER-2/neu gene – orange signals, centromere 17 – green signals*

Table 1. **Distribution of histopathological examination results that were different from the preoperative clinical staging, according to TNM classification**

TNM status	*Group 1 (n=31)*	*Group 2 (n=11)*	*Group 3 (n=6)*	*Total (n=48)*
T2N0M0	1 (3%)	3 (27%)	1 (17%)	5 (10%)
T1N1M0	0 (0%)	2 (18%)	2 (33%)	4 (8%)
T2N1M0	0 (0%)	1 (9%)	1 (17%)	2 (4%)
T2N2M0	0 (0%)	0 (0%)	1 (17%)	1 (2%)
Total	1 (3%)	6 (55%)	5 (83%)	12 (25%)

Table 2. **Number of total and metastatic, and ratio of metastatic to total axillary lymph nodes in the three groups**

	Group 1 (n=31)	*Group 2 (n=11)*	*Group 3 (n=6)*
Total dissected nodes	30±7	26±4	24±7
Metastatic	5±2	6±3	20±7
Metastatic/total ratio	17%	23%	83%

after PMAD were 2.2±0.5 cm (range, 0.5-4 cm) and 2.5±0.7 cm in all patients, respectively.

Patients included in the study had clinical $T_{1-2}N_0M_0$ invasive ductal carcinoma diagnosis. According to post-PMAD histopathological examinations, different staging from this preoperative clinical staging was obtained in some of the patients (*Table 1*). Adjuvant chemotherapy regimens were planned according to the histopathological staging. Based on histopathological results, one patient of group 1 (3%), 6 patients of group 2 (55%), and 5 patients of group 3 (83%) received chemotherapy (P=0.04).

The presence of axillary metastases in each group is shown in *Figure 2*. The axillary metastasis rate was highest in group 3 (P=0.002). The mean number of total lymph nodes dissected from axilla was similar in the three groups (P>0.05, *Table 2*). The mean number of metastatic axillary lymph nodes as well as the ratio of metastatic lymph nodes to dissected total lymph nodes were highest in group 3 (P=0.001 and P=0.003, respectively). In addition, the presence of axillary micrometastases was 3% (1/31) in group 1, 9% (1/11) in group 2, and, 50% (3/6) in group 3, the percentage being highest in group 3 (P=0.008).

Through the 5-year follow-up period, distant metastasis rates were 16% (5/31), 18% (2/11), and, 17% (1/6) in groups 1, 2, and 3, respectively. Local recurrence rates in the same period were 6% (2/31) in group 1, 9% (1/11) in group 2, and 33% (2/6) in group 3. With respect to distant metastasis in a 5-year follow-up period, all the groups were similar, while the rate of local recurrence was the highest in group 3 (P=0.04).

In the current patient population, estrogen receptor (ER) expression, assessed previously, seemed to be associated with decreasing signal ratios. While 35.5% and 27.3% of patients in group 1 and 2, respectively, had positive ER status, all patients in group 3 had ER-negative results (P=0.03). All patients with ER-positive tumor received routine antiestrogen therapy for a period of five years.

Histopathologically, irregular growth pattern of the tumor was observed in 54% (17/31), 60% (6/11), and 100% (6/6) of the patients in groups 1, 2 and 3, respectively (P=0.04). Hypercellularity of the tumor was highest in group 3 (P=0.01). EIC ratios were 26% (8/31) in group 1, 18% (2/11) in group 2, and 67% (4/6) in group 3 (P=0.04). The level of DR and lymphocyte infiltration did not show significant difference between groups. The presence of LVI was 13% (4/31) in group, 9% (1/11) in group 2, and 50% (3/6) in group 3 (P=0.01). In this study, our results showed that the HER-2/*neu* signal increased with worsening of clinical and histopathological malignancy criteria.

Discussion

HER-2/*neu* is a highly promising target for specific anticancer therapy, and there has been an increasing demand for its analysis in archival and current breast cancer specimens. Several methods have been used in these analyses,

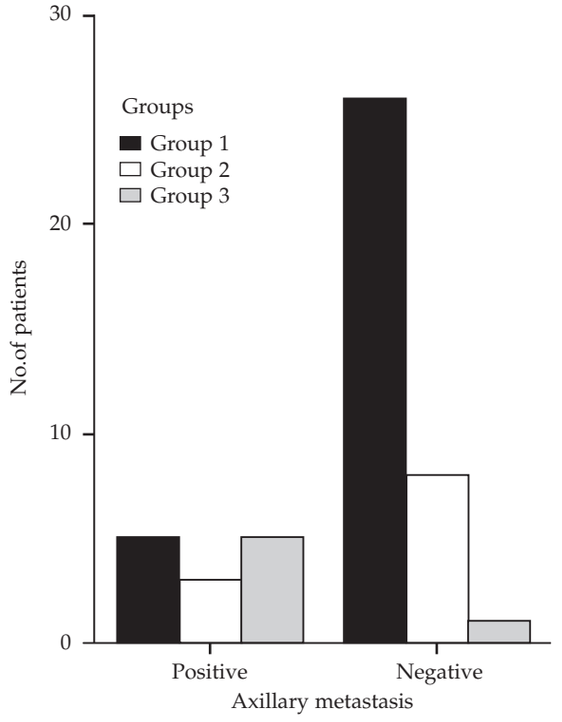

Figure 2. *Number of patients with metastatic axillary lymph nodes in groups 1, 2, and 3. The highest rate of metastasis was in group 3 (P = 0.002).*

and FISH is currently considered to be the most specific and sensitive method for detection of amplification of oncogenes in human tumor samples.[1] Interphase FISH is a highly sensitive marker in tumor studies; it is especially applicable to samples obtained from conventional cytogenetic procedures and paraffin-embedded, formalin-fixed tissue sections.[8] It is possible to analyze genetic aberrations present even in a small percentage of tumor cells by interphase FISH.

HER-2/*neu* status in breast cancer patients is the only eligibility criterion for trastuzumab treatment. This emphasizes the importance of detection methods. In a previous study, Sumita *et al*. considered FISH as a gold standard for determination of HER-2/*neu* status in breast cancer.[9] In multivariate analyses, HER-2/*neu* has been reported to be an independent prognostic factor. In lymph node-negative patients, HER-2/*neu* was not reported to be a prognostic indicator.[10] In a recent review by Bartlett *et al*. a comparison of diagnostic FISH and IHC approaches have been made in patients with discordant FISH and IHC results, enabling a determination of the discriminatory power of these tests.[11] In a study, patients with FISH-amplified/IHC-negative tumors had a 10-year survival of 50% which was similar to that of the patients positive by both methods. Conversely, patients with FISH-negative/IHC-positive tumors exhibited a 10-year survival similar to that of patients negative for both FISH and IHC.[12] In the present study, we aimed both to detect HER-2/*neu* status of the patients and to define the association between clinical and histopathological findings and HER-2/*neu* amplification.

The association between poor prognosis and HER-2/*neu* amplification is known.[3] In our patients, we determined axillary metastasis ratio, the number of axillary metastatic lymph nodes, and axillary micrometastases. We concluded that these parameters were closely related to increasing signal ratios. This is confirmed by the highest HER-2/*neu* signal in relation to both the highest axillary metastatic lymph node numbers and axillary micrometastases occurring in group 3. These findings support previous reports about considering HER-2/*neu* as a biological marker of tumor aggressiveness. In their review, Ross *et al*. reported that of 81 studies evaluated, 73 reports that amplification or overexpression of HER-2 predicted breast cancer outcome.[12,13]

We observed the highest local recurrence ratio in group 3. We did not observe any difference between the three groups when the distant metastasis ratio over a 5-year follow-up period was examined.

An inverse relationship between ER positivity and HER-2/*neu* amplification has been reported.[14] Activation of HER-2 receptor in breast cancer cells leads to the estrogen-independent growth of the cells and to estrogen- and tamoxifen-insensitive development of tumors. Also, HER-2 receptor overexpression in estrogen-dependent tumor cells promotes downregulation of ER and delayed autoregulation in suppression of ER transcripts; 50% of HER-2/*neu*-positive tumors have been found to be ER-positive in contrast to the 75% ratio in the whole population.[15] Our results support these reports as we also had findings of decreased signal numbers with ER expression. This finding is important as it has been reported that hormonal insensitivity of HER-2/*neu*-positive tumors could result from ER negativity rather than HER-2/*neu* positivity.[16] The prognosis of our ER-negative patients treated with tamoxifen did not change in the case of synchronous HER-2/*neu* overexpression, which is consistent with the study of Elledge et al. [16]

We observed a relationship between the histopathologically defined tumor pattern and HER-2/*neu* amplification. In group 3, the IGP, HC, and EIC ratios were significantly higher than in the other groups. Lymphocyte infiltration and DR did not reveal a significant difference between the three groups; these findings partly support previous reports.[11]

According to our findings, in addition to the higher lymph node metastasis rate a higher local recurrence rate can be expected in group 3. However, similar distant metastasis rates were found in each group. It is obvious that the number of patients included in the study is not sufficient to reach a clear conclusion, and in addition the findings of 10- and 20-year follow-up periods need to be considered.

We conclude that both clinical and histopathological findings, as well as HER-2/*neu* status, should be considered in breast cancer patients when planning therapy regimens and during the follow-up of patients. Our results show that HER-2/*neu* signal ratio increases with worsening of clinical and histopathological malignancy criteria. However, these findings need to be confirmed by further investigations.

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