Comparison of Her-2, EGFR and Cyclin D1 in Primary Breast Cancer and Paired Metastatic Lymph Nodes: An Immunohistochemical and Chromogenic In Situ Hybridization Study

The significant advance in the development of molecular-targeting drugs has made an evaluation of Her-2, EGFR, and cyclin D1 an important clinical issue in breast cancer patients. This study compared the Her-2, EGFR, and cyclin D1 status of primary tumors as well as their matching lymph node metastases using immuno-histochemistry (IHC) and chromogenic in situ hybridization (CISH) in 73 breast cancer patients. Her-2, EGFR, and cyclin D1 protein showed a concordance between the primary lesion and the metastatic regional lymph nodes in 82%, 90%, and 63%, respectively. CISH also revealed 92%, 93%, and 85% concordance in the gene amplification status of Her-2, EGFR, and cyclin D1, showing a reasonable agreement between primary tumors and metastatic regional lymph nodes. Although a statistically significant agreement was found in Her-2 expression, a relatively high discordance rate (18%) raises a little concern. Our findings suggest that the Her-2 status can be reliably assessed on primary tumor but a possible difference can be found in Her-2, EGFR, and cyclin D1 status between the primary and the metastatic sites and this possibility should be concerned in patients considering molecular targeted therapy or patients with progress of disease.

Key Words : *CISH; Her-2; EGFR; Cyclin D1; Immunohistochemistry*

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INTRODUCTION

Breast cancer is one of most common malignancies in women. It has been suggested that changes in many genes encoding growth factors, growth factor receptors and other regulators of proliferation might play a role in the tumori-genesis and the response to breast cancer treatment (1). Recently, an amazing improvement, which focused on the development of molecular-targeting drugs, was achieved in anti-cancer therapy. Clinical trials are currently testing monoclonal antibodies and tyrosine kinase inhibitors against Her-2 and EGFR, and other molecular strategies against their down-stream targets including cyclin D1 (2, 3). Therefore, it is important to evaluate the overall Her-2, EGFR, and cyclin D1 status in targeting the gene product.

Molecular targeted therapy has been suggested in the treatment of metastatic breast cancer. Trastuzumab is a high-affinity, humanized, anti-Her-2 antibody for the treatment of advanced breast carcinoma, particularly metastasis. However, it was shown that less than half of patients with a high level of Her-2 expression respond to Trastuzumab treatment (4, 5). One explanation might be heterogeneity in the expression of Her-2 between primary and metastatic tumor cells (6). It is also unclear whether protein overexpression or gene

amplification is more predictive in the response to the novel modalities, such as small molecular inhibitors (7). Furthermore, there is no agreement as to whether the molecular status should be assessed in primary tumors or metastases.

A change in the biological parameters is believed to occur in the early stages of the progression of breast cancer and might be maintained throughout cancer progression (8, 9). It is very important to determine if the biological parameters in metastatic regional lymph nodes are similar to that in the primary breast cancer because therapy is indicated for patients with synchronous metastatic regional lymph nodes of breast cancer. In breast cancer, the axillary lymph nodes are often the first sites to harbor metastases, and metastasis in this region is the most powerful prognostic factor for breast cancer patients (10). However, in most studies, the status of the targeted molecules is determined on the primary tumor, and there are a few reports regarding the comparison of Her-2 status between the primary and the metastatic lymph nodes (11-17). Moreover, there have been no comprehensive studies evaluating gene amplification and protein expression in Her-2, EGFR, and cyclin D1 in the metastatic lesions. To the best of our knowledge, this is the first study to compare Her-2, EGFR, and cyclin D1 status of primary breast tumors with their metastatic lymph nodes using immunohistochemistry (IHC)

and chromogenic in situ hybridization (CISH) techniques.

In this study, we compared the protein expression and gene amplification of Her-2, EGFR, and cyclin D1 between primary breast cancer and the corresponding metastatic lymph node using IHC and chromogenic CISH techniques, and examined their association with the recurrence-free survival.

MATERIALS AND METHODS

Sample collection

Seventy-three cases of breast tumor from the surgical pathology files of the Department of Pathology at Samsung Medical Center were investigated. The inclusion criteria were a histopathological diagnosis of invasive breast carcinoma with paired metastatic tumors in the lymph nodes, the availability of clinical data, and the availability of paraffin-embedded tissue specimens. All the patients had undergone surgery, and had received neither chemotherapy nor radiotherapy before the surgical resection. Table 1 shows the main characteristics of the primary tumors. Breast cancer specimens were reviewed using morphologic criteria according to the WHO classification of breast cancer. All the patients were women and the mean age was 47.77 yr (range, 28-70). The mean number of metastatic lymph nodes was 4.95 (range 1-27, median 3.00). Raw survival data were obtained from patients' chart. The mean follow-up time was 45.5 (range, 1-63) months. Recurrence-free survival was expressed as the number of months from diagnosis to the occurrence of an event (local recurrence/metastasis).

Assembly of the tissue microarrays

The surgical specimens were fixed in 10% buffered formalin, processed, and embedded in paraffin using the standard protocol. The representative areas on the hematoxylin and eosin (H&E)-stained sections were carefully selected and marked on individual paraffin blocks. Two tissue cores (1-mm diameter) were obtained from each primary carcinoma sample and lymph node specimen, respectively. The tissue cores were arrayed in a recipient paraffin block according to the manufacturer's instructions.

Chromogenic in situ hybridization

The sections (4 μm) were cut from the array blocks, transferred to adhesive-coated slides and air-dried in an incubator for 16 hr at 65°C. The slides were deparaffinized in xylene and graded ethanol followed by washing in phosphate-buffered saline (PBS). Microwave antigen retrieval was performed in 100-mM Tris/50 mM EDTA, pH 7.0 at 93°C for 15 min. After washing three times in PBS for 2 min each, the slides were incubated in Digest-All 3 (Zymed, San Francisco, CA, U.S.A.) for 3 min at 37°C. After digestion, the slides were washed in PBS, fixed in 10% phosphate-buffered formalin for 1 min, and dehydrated through a 70%, 85%, 95%, and 100% ethanol series at room temperature. The digoxigenin-labeled Her-2/*neu* (Zymed), EGFR (Zymed), and cyclin D1 probes (Zymed) were placed on each slide, and a coverslip was applied and sealed with rubber cement. The slides were denatured for 3 min at 93°C and incubated at 37°C overnight in a humidified chamber. A posthybridization wash was performed the next day, and immunodetection was performed at room temperature with a CISH polymer detection Kit (Zymed) and 3,3′-diaminobenzidine (DAB) as the chromogen. The slides were counterstained with hematoxylin and mounted.

The CISH results were evaluated by optical microscopy at low- and high- magnification, and scored as previously described (18). High-level gene amplification was defined as more than 10 discrete copies per nucleus or as large gene copy clusters (observed as confluent masses containing more than 10 signals) in more than 50% of the nuclei evaluated. Low-level amplification was defined as 6 to 10 copies per nucleus in more than 50% of cells. Unaltered gene copy was defined as 1 to 5 copies per nucleus.

Immunohistochemistry

After deparaffinization and rehydration, 4-μm thick sections on silane-coated slides were heat-pretreated in a citrate buffer (pH 7.3 at 92°C in microwave oven) and examined by immunostaining using specific antibodies against Her-2

Table 1. Clinicopathologic findings of the 73 primary breast tumors

Characteristics	No. of patients (%)
Age	
<40	17 (23.3)
40-49	25 (34.2)
>50	31 (42.5)
Tumor size	
<2 cm	18 (24.7)
2-4 cm	37 (50.7)
≥4 cm	18 (24.7)
No. of positive nodes	
1-3	43 (58.9)
4-7	19 (26.0)
>7	11 (15.1)
Nuclear grade	
1	2 (2.7)
2	37 (50.7)
3	34 (46.6)
Histologic grade	
I	6 (8.2)
II	40 (54.8)
III	27 (37.0)

(CB11; Novocastra Laboratories, Newcastle, U.K.), EGFR (Novocastra Laboratories), and cyclin D1 (P2D11F11; Novocastra Laboratories). The avidin-biotin technique was applied using DAB for visualization and hematoxylin for nuclear counterstaining.

Her-2 and EGFR immunoreactivity was assessed using the following scoring approach: 0, no immunoreactivity or immunoreactivity in <10% of tumor cells; 1+, faint weak and incomplete staining of >10% of tumor cells; 2+, weak to moderate complete membrane immunoreactivity in >10% of tumor cells; 3+, moderate to strong complete membrane immunoreactivity in >10% of tumor cells. The cyclin D1 protein is located in the cellular nucleus of the cells. The Allred score was used to evaluate the cyclin D1 immunostain (19). The proportion and intensity scores of cyclin D1 staining were added, and the staining was assessed as either: 0, no immunoreactivity; 1+, total scores 1-2; 2+, total scores 3-5; 3+, total scores 6-8. Cases interpreted as 0 or 1+ were considered negative, and cases interpreted as 2+ or 3+ were considered positive.

Statistical analysis

The agreement of Her-2, EGFR, or cyclin D1 status between primary tumors and matched metastatic lymph nodes is expressed both by concordance and by the Cohen κ coefficient. The relation between the kappa value and the level of agreement was suggested by Landis and Koch (20), with κ values of 0.00-0.20 indicating slight agreement, 0.21-0.40 indicating fair agreement, 0.41-0.60 indicating moderate agreement, 0.61-0.80 indicating substantial agreement, and 0.81-1.00 indicating almost perfect agreement. The Kaplan-Meier method was used to estimate recurrence-free survival. A log-rank test was applied to examine the relationship between IHC or CISH data and recurrence-free survival. p values less than 0.05 were considered to be statistically significant. Calculations were done with SPSS 11.0 (SPSS, Inc., Chicago, IL, U.S.A.).
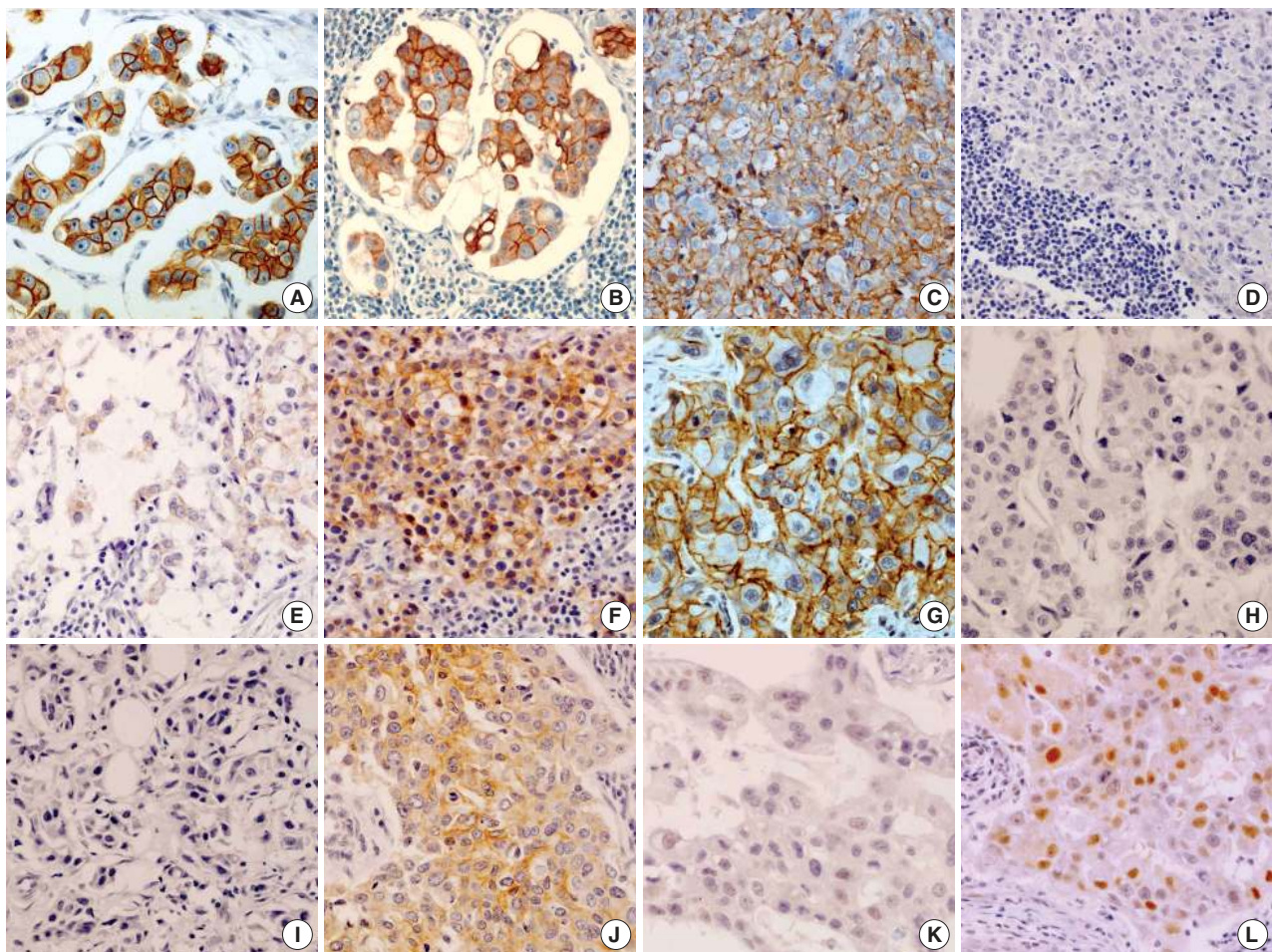

Fig. 1. Comparison of immunostaining patterns of Her-2, EGFR and cyclin D1 in primary tumor and the corresponding metastatic lymph node (LN) (×200). 3+/3+ expression of Her-2 in primary tumor (A) and LN (B); 2+/− expression of Her-2 in primary tumor (C) and LN (D); 1+/2+ expression of Her-2 in primary tumor (E) and LN (F), 3+/− expression of EGFR in primary tumor (G) and LN (H); −/2+ expression of EGFR in primary tumor (I) and LN (J); −/3+ expression of cyclin D1 in primary tumor (K) and LN (L).

RESULTS

Table 1 shows the clinical and pathologic characteristics of 73 patients. Table 2, 3, and 4 show the comparisons of Her-2, EGFR, and cyclin D1 status between the primary lesions and matching metastatic regional lymph nodes, respectively. Comparative features of IHC and CISH in the primary lesions and paired metastatic regional lymph nodes are depicted in Fig. 1 and 2.

Her-2 status in primary and paired metastatic carcinoma in lymph node (Table 2)

Using IHC, 37.0% of the primary tumors and 32.8% of the corresponding metastatic lymph node specimens over-expressed Her-2. Among 73 cases, 60 (82.2%) cases showed concordant expression between the primary tumors and metastatic lymph nodes. Nine cases of 13 discordant cases

showed one-level difference of staining score. Regarding CISH, 41.6% of the primary tumor specimens and 36.1% of the corresponding metastatic lymph node specimens were amplified. Only 6 (8.3%) of 73 cases showed discordant results when the primary tumors and their metastatic lymph nodes were compared. Four discordant cases were low-amplified lesions. IHC and CISH for HER2 status showed moderate or perfect agreement with a κ coefficient of 0.609 ($p < 0.001$) and 0.824 ($p < 0.001$), respectively.

EGFR status in primary and paired metastatic carcinoma in lymph node (Table 3)

Seventy cases were evaluated for EGFR IHC. EGFR-positivity was observed in 4 primary tumors (5.7%) and 5 metastatic lymph nodes (7.1%). CISH analysis of the primary and metastatic samples in 71 cases demonstrated 7.0% and 5.6% EGFR amplification, respectively. The EGFR status
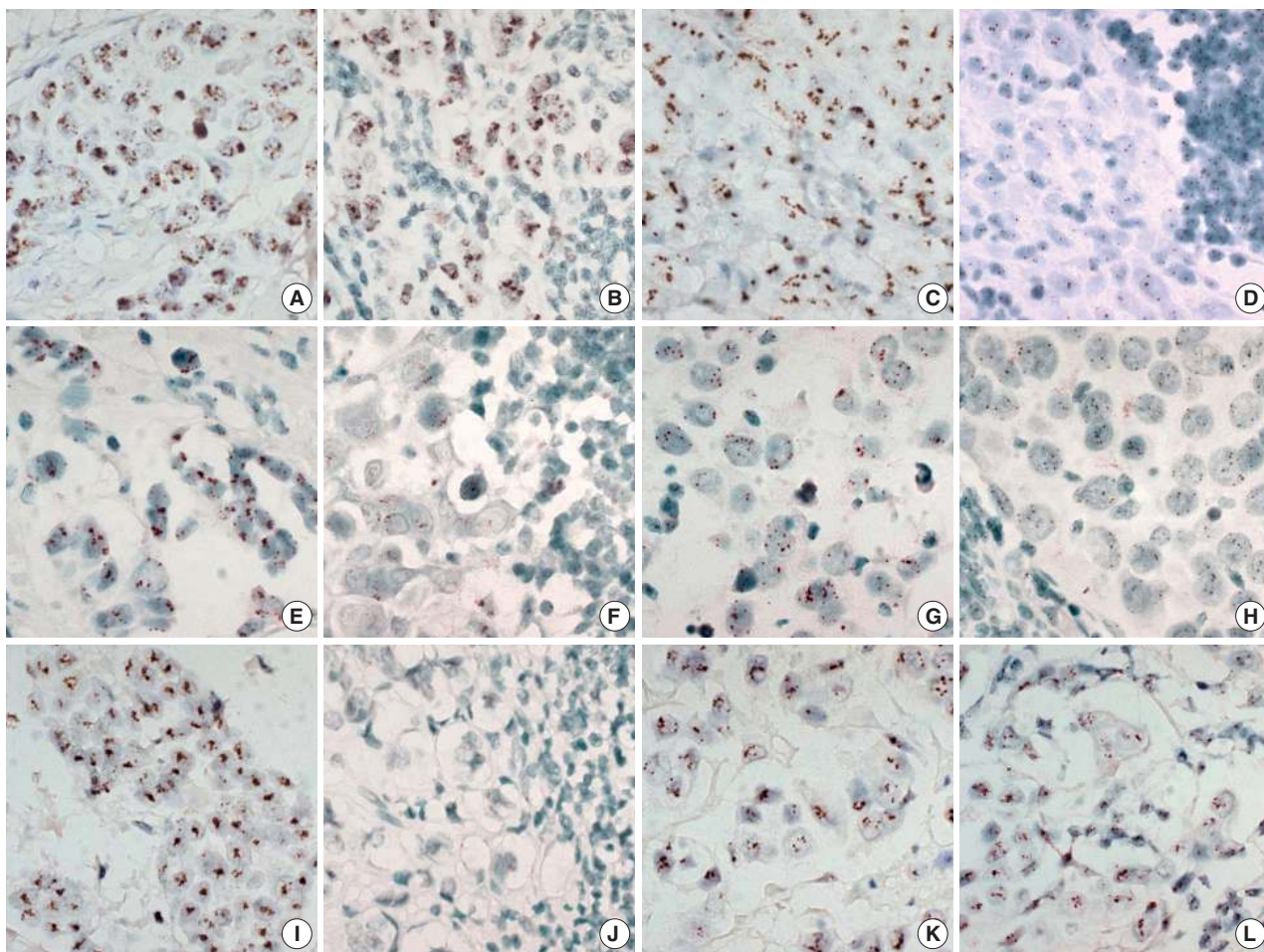

Fig. 2. Comparison of gene amplification patterns of Her-2, EGFR and cyclin D1 in primary tumor and the corresponding metastatic lymph node (LN) (×400). High-level amplification/high-level amplification of Her-2 in primary tumor (A) and LN (B); high-level amplification/no amplification of Her-2 in primary tumor (C) and LN (D); high-level amplification/no amplification of EGFR in primary tumor (E) and LN (F); low-level amplification/low-level amplification of EGFR in primary tumor (G) and LN (H); high-level amplification/no amplification of cyclin D1 in primary tumor (I) and LN (J); high-level amplification/high-level amplification of cyclin D1 in primary tumor (K) and LN (L).

Table 2. Her-2 status in the primary and paired metastatic carcinoma in the lymph node

Variable	Immunohistochemistry (primary/metastatic tumor) (n=73)				CISH (primary/metastatic tumor) (n=73)			
	+/+	+/-	-/+	-/-	+/+	+/-	-/+	-/-
No. of patients	19 (26.0%)	8 (11.0%)	5 (6.8%)	41 (56.2%)	25 (34.7%)	5 (6.9%)	1 (1.4%)	41 (56.9%)
Concordance	82.2%				91.7%			
κ coefficient	0.609 (p<0.001)				0.825 (p<0.001)			
No. of patients with recurrence	3 (25%)	1 (8.3%)	1 (8.3%)	7 (58.3%)	4 (33.3%)	0	0	8 (66.7%)

CISH, chromogenic in situ hybridization.

Table 3. EGFR status in the primary and paired metastatic carcinoma in the lymph node

Variable	Immunohistochemistry (primary/metastatic tumor) (n=70)				CISH (primary/metastatic tumor) (n=71)			
	+/+	+/-	-/+	-/-	+/+	+/-	-/+	-/-
No. of patients	1 (1.4%)	3 (4.3%)	4 (5.7%)	62 (88.6%)	2 (2.8%)	3 (4.2%)	2 (2.8%)	64 (90.1%)
Concordance	90.0%				93.0%			
κ coefficient	0.169 (p=0.153)				0.407 (p=0.001)			
No. of patients with recurrence	1 (9.1%)	0	1 (9.1%)	9 (81.8%)	0	2 (16.7%)	0	10 (83.3%)

CISH, chromogenic in situ hybridization.

Table 4. Cyclin D1 status in the primary and paired metastatic carcinoma in the lymph node

Variable	Immunohistochemistry (primary/metastatic tumor) (n=73)				CISH (primary/metastatic tumor) (n=71)			
	+/+	+/-	-/+	-/-	+/+	+/-	-/+	-/-
No. of patients	30 (41.1%)	8 (11.0%)	19 (26.0%)	16 (21.9%)	4 (5.6%)	8 (11.3%)	3 (4.2%)	56 (78.9)
Concordance	63%				84.5%			
κ coefficient	0.250 (p=0.025)				0.339 (p=0.003)			
No. of patients with recurrence	3 (25%)	2 (16.7%)	5 (41.7%)	2 (16.7%)	1 (8.3%)	1 (8.3%)	1 (8.3%)	9 (75%)

CISH, chromogenic in situ hybridization.

examined with IHC and CISH showed 90.0% (63 out of 70 cases) and 92.9% (66 out of 71 cases) concordance between the primary breast lesions and metastatic lymph nodes, respectively. Changes in EGFR expression were from 2+ or 3+ to 0. The κ coefficient rates were 0.169 (p=0.153) and 0.407 (p=0.001) in IHC and CISH, respectively, showing moderate agreement by CISH analysis.

Cyclin D1 status in primary and paired metastatic carcinoma in lymph node (Table 4)

For 73 cases examined by IHC, the cyclin D1 protein was expressed in 52.1% of primary lesions and in 67.1% of metastatic lymph nodes. Concordant cyclin D1 IHC results were found in 43 cases (63.0%). Nineteen (70.4%) out of 27 discordant cases showed greater expression in the metastatic sites. Seventy-one paired samples were simultaneously evaluated for CISH. There were 12 (16.9%) and 7 cases (9.8%) with cyclin D1 amplification in both primary tumors and metastatic lymph nodes, respectively. CISH analysis of the primary and metastatic samples demonstrated 84.5% (60

out of 71 cases) concordance. In statistical analysis, the κ coefficient was 0.250 (p=0.025) and 0.339 (p=0.003) in IHC and CISH, respectively. CISH analysis showed fair agreement between the primary and paired metastatic tumors.

Recurrence-free survival according to Her-2, EGFR, and cyclin D1 status in primary mass and lymph node

Among 64 cases with follow-up, 12 patients had experienced an event (local recurrence/metastasis) and one patient with recurrence died of disease. Protein expression and gene amplification of Her-2, EGFR, and cyclin D1 in primary tumor and metastatic lymph node was not associated with disease-free survival. However, there was a significant decrease in disease-free survival in patients with EGFR amplification in primary tumor and no EGFR amplification in metastatic tumor (p=0.0454, Fig. 3).

Regardless of Her-2 status in metastatic sites, three recurrent cases with Her-2 overexpression in primary tumors had received Trastuzumab treatment and have been alive without progress of disease. On the other hand, one patient who
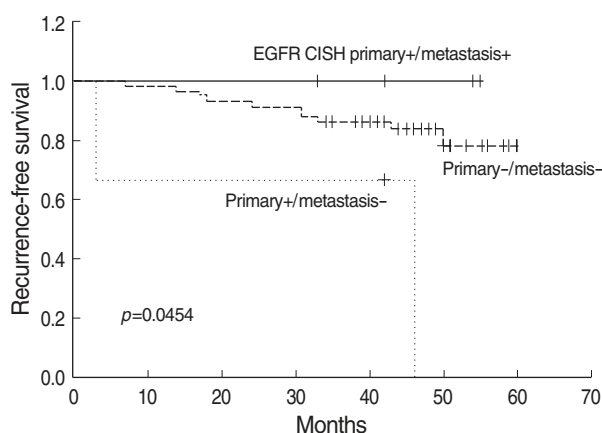
Fig. 3. Recurrence-free survival according to EGFR amplification. Disease-free survival is significantly low in patients with EGFR amplification in primary tumor and no EGFR amplification in metastatic tumor (p=0.0454).

had Her-2 protein overexpression in metastatic lymph node but no immunoreactivity in primary tumor, has been suffering from progressive metastasis (Table 2).

DISCUSSION

The important role of targeted therapy against molecules contributing to tumor development, progression, and metastasis has attracted considerable attention. Molecular-targeting drugs against Her-2, EGFR, and cyclin D1 are a major concern in breast cancer patients (2, 3). These molecules are mainly evaluated at the primary site and there is little data available regarding the markers between the primary tumor and the corresponding metastases. However, the death of metastatic cells is the main goal of treatment in a metastatic setting. These cells may be biologically different from the primary tumor, which has implications for the clinical management of breast cancer. Therefore, it is essential to determine if there is homogeneous biological marker expression between primary tumor and its metastatic sites. In breast carcinoma, a lymph node metastasis is an indicator of a poor prognosis and represents the beginning of the progression from local to systemic disease, which poses serious challenges with regard to treatment. Although lymph node metastases should not be assumed to be biologically equivalent to distant metastases, it has been hypothesized that there are subtle genetic difference between the primary tumor and lymph node metastases (21).

Her-2 is the most promising biological marker in terms of predictive value for breast cancer treatment. Although not complete, a number of studies have reported a high level of consistency in the Her-2 status between primary tumors and locoregional metastases using IHC and fluorescence in situ hybridization (FISH) (11-17). Most data have shown a

good overall concordance of Her-2 status between primary and metastatic tumors (11, 13, 14, 17). However, some data have shown disagreement in up to 20% of tumors (22). The present study demonstrated relatively good accordance of the Her-2 status between the primary lesion and the metastatic regional lymph node (82% and 92% when analyzed by IHC or CISH, respectively). In particular, Her-2 amplification showed strong agreement compared with the moderate agreement of Her-2 overexpression. These findings suggest that metastatic breast carcinoma in lymph nodes generally overexpresses Her-2 in a manner similar to the corresponding primary tumors, and Her-2 gene is stable in a metastasis. However, the discordance rate of Her-2 overexpression in the present study was relatively high (18%), and this fact could not be completely ignored. In addition, fascinating findings were observed in the follow-up results of the stratified subgroups according to the Her-2 status in primary and metastatic tumors. Without reference to Her-2 status in metastatic sites, three recurrent cases with Her-2 overexpression in primary tumors and Trastuzumab treatment for metastatic disease have been alive with no progress of disease by this time. On the other hand, one patient who had Her-2 protein overexpression in the metastatic lymph node but no immunoreactivity in primary lesion, has been suffering from progressive metastatic disease during follow-up and she did not receive treatment with Trastuzumab as her tumor was negative for Her-2. Although present study included small number of informative cases, our results suggests that Her-2 evaluation in metastatic sites can benefit from treatment with Trastuzumab when Her-2 is negative in the primary tumor.

There have been few studies that have evaluated EGFR or cyclin D1 status in metastatic tumors (23-25). Tsutsui et al. demonstrated a strong concordance rate (92%) of EGFR immunoreactivity between the primary breast cancers and matching regional lymph nodes (25). However, the most recent study demonstrated that EGFR expression is not stable during metastatic progression in a significant proportion of primary lung cancer (26). The present study demonstrated insignificant correlation of EGFR protein expression (p=0.153) between the primary lesions and the metastatic regional lymph node in spite of the high concordance observed (90.0%). On the other hand, the *EGFR* gene in the primary and corresponding metastatic lesions revealed moderate agreement even though showed similar concordance to EGFR protein expression. This statistical finding might be induced by low positive rate of EGFR expression and further studies with larger numbers of patients are needed to verify our results. In the present study, the positive rate of EGFR staining seems to be lower than that of previous reports, but is consistent with the result of Bhargava et al. using the same scoring system (27).

Regarding cyclin D1, Hao et al. demonstrated that 61 out of 86 paired samples exhibited no difference in cyclin D1 expression (23). In 9 out of 86 cases, the cyclin D1 expres-

sion level was higher in the metastasis samples, whereas the expression level was lower in the metastases of 16 out of 86 cases. They reported a trend toward decreasing cyclin D1 expression in metastases despite the lack of statistical significance. In this study, discordant results were observed in 27 out of 73 cases by IHC and 11 out of 71 cases by CISH. The frequency of cyclin D1 overexpression was higher in the metastases but the level of amplification showed fair agreement between primary and corresponding metastatic lymph nodes. In this study, the cyclin D1 protein was overexpressed more frequently than amplification, again within the range of frequencies previously described for breast cancer patients (28, 29). Although the immunohistochemical reactivity of cyclin D1 has been evaluated by various methods, cyclin D1 overexpression is observed in up to 68% of primary breast cancers, and the frequency of cyclin D1 protein overexpression exceeded the frequency of DNA amplification (30-32). The significance of cyclin D1 protein or gene as prognostic factors for survival in breast cancer patients is still controversial (33). Although cyclin D1 overexpression has been reported to indicate resistance to tamoxifen treatment, cyclin D1 amplification was reported to be a more powerful predictor of a successful tamoxifen treatment particularly in node-positive patients (34). Therefore, the overall evaluation of the cyclin D1 status using IHC and FISH would be helpful in selection of patients who may benefit from targeted therapy.

IHC and FISH are the preferred methods for evaluating protein overexpression and gene amplification because of their ability to distinguish the tumor's morphological features. The critical advantages of IHC over FISH are the lower cost and easy automation. Therefore, IHC can be performed in most pathology laboratories without the requirement of a fluorescence microscope. Compared with IHC, FISH provides an accurate assessment of the gene copy number and is the universally accepted gold standard for evaluating gene amplification. However, this technique is not ideally suited for tissue microarrays because it is difficult and time consuming to navigate through the tissue microarray section under a fluorescent microscope. CISH was recently introduced to visualize gene alteration with an IHC-like peroxidase reaction and has been reported to be a good substitution for FISH. This method only requires an ordinary microscope rather than an expensive fluorescence microscope. The CISH signals are more familiar to the pathologists and morphological detail is more easily appreciated in bright field and therefore quicker to observe in tissue microarray slides. In this study, CISH was performed successfully on tissue microarray sections without any technical problem. Our study demonstrates that CISH is a useful tool to easily assess Her-2, EGFR, and cyclin D1 genes in surgical laboratory.

In this study, tissue microarray (TMA) techniques allowed the rapid and high throughput of IHC and CISH. TMA has been used for IHC and FISH techniques, and is a highly reliable method through avoiding day-to-day variability in the staining conditions as well as in the interpretation of the staining results. However, the study using TMA may have the limitations of the representative of the selected samples because of tumor heterogeneity. This can be avoided by punching two or more cores from different areas (35). We made TMA blocks with two tissue cores (1-mm diameter) from each primary carcinoma sample and lymph node specimen, respectively. We think that two cores of 1-mm diameter specimen are representative to perform IHC or CISH to some degree, although we cannot completely exclude the limitation of the study using TMA.

Most previous reports have shown a good concordance rate of Her-2 status between primary and metastatic tumors. Compared the previous reports based on the status of samplings such as whole paraffin blocks or TMA samples, the concordance rate of Her-2 status between the primary and the metastatic sites is not different (80-100% in whole paraffin specimen [11-14, 17, 22, 25] vs. 93% in TMA specimen [16]). Because a study that revealed a relatively high discordance rate of 20% was performed using whole paraffin specimen (22), lack of concordance in the study seems to result from clonal selection or genetic drifts in metastatic sites rather than tumor heterogeneity.

In conclusion, our results suggest that Her-2 status tends to be stable during metastatic progression in a significant proportion of breast cancer but a possible difference in Her-2, EGFR, and cyclin D1 status between the primary and the metastatic sites would be concerned for breast cancer patients considering molecular targeting therapy. Since it is not yet unclear which is more predictable in the response of molecular targeting therapy, protein overexpression or gene amplification, CISH along with IHC may be implemented in routine assessment of Her-2, EGFR and cyclin D1 status for breast cancer patients.

ACKNOWLEDGMENT

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