p185HER-2/neu and p21CIP1/WAF1 Expression in Primary Tumors and Lymph Node Metastases in Non-small Cell Lung Cancer

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p185HER-2/neu, a tyrosine kinase receptor, is one of the target molecules for cancer therapy, and its expression may reduce the sensitivity of tumor cells to anti-cancer drugs. p21CIP1/WAF1 is a cyclin-dependent kinase inhibitor, and its expression may also be involved in chemoresistance. Non-small cell lung cancer (NSCLC) is a potentially systemic disease, and systemic therapies play an important role in its treatment. However, there have been no studies comparing the expression of these molecules between primary and metastatic tumors. We investigated the expression of p185HER-2/neu and p21CIP1/WAF1 in 57 paired samples of primary NSCLC tumors and corresponding lymph node metastases by immunohistochemistry. Expression of each of p185HER-2/neu and p21CIP1/WAF1 was highly correlated between primary tumors and lymph node metastases, and similar correlations were also obtained when adenocarcinoma and squamous cell carcinoma cases were analyzed individually. However, we failed to detect any correlation between p185HER-2/neu and p21CIP1/WAF1 expression. Our results suggested that expression of both p185HER-2/neu and p21CIP1/WAF1 is concordant between primary and metastatic tumors.

Key words: p185HER-2/neu — p21CIP1/WAF1 — Lymph node metastases — Non-small cell lung cancer

p185HER-2/neu (also known c-erbB-2) is encoded by the HER-2/neu/c-erbB-2 proto-oncogene that is amplified or overexpressed in about 30% of human cancers including breast, ovarian, gastric, colon, and non-small cell lung cancers. This molecule is a tyrosine kinase receptor that belongs to the erbB receptor family and shows partial homology to epidermal growth factor receptor. The anti-tumor activity of anti-HER-2 monoclonal antibody therapy against p185HER-2/neu-overexpressing tumors has been reported.1–4 However, such tumors may show reduced sensitivity to various anti-cancer drugs.5–7

p21CIP1/WAF1, a product of the WAF1/CIP1/SDI1/mda-6 gene, is a cyclin-dependent kinase inhibitor, and can be induced through both p53-dependent and independent pathways. p21CIP1/WAF1 expression is also associated with anti-cancer drug sensitivity.8,9 Recently, it was reported that p21CIP1/WAF1 may be involved in chemoresistance of p185HER-2/neu-overexpressing tumor cells.10

Non-small cell lung cancer (NSCLC) is a potentially systemic disease, and therapies effective against not only primary, but also metastatic tumors are needed to achieve longer survival of patients. The features and the clinical relevance of p185HER-2/neu and p21CIP1/WAF1 have been well clarified in NSCLC. However, these studies relied mainly on primary tumor assessment, and whether expression of the above two molecules differs between primary and secondary metastatic tumors has not been elucidated. In addition, there have been no studies of the relationship between p185HER-2/neu and p21CIP1/WAF1 expression. In the present study, we investigated the expression of p185HER-2/neu and p21CIP1/WAF1 in 57 patients with NSCLC by immunohistochemistry, comparing the primary tumors with the corresponding lymph node metastases.

MATERIALS AND METHODS

Patients and tissue samples The tissues of primary tumors and metastatic lymph nodes were obtained from 57 (44 males and 13 females) consecutive patients with NSCLC who underwent surgical treatment over a period of 10 years at the Second Department of Surgery, Nagoya City University Hospital. The numbers of patients with adenocarcinoma, squamous cell carcinoma, adenocarcinoma cell carcinoma and large cell carcinoma were 27, 26, 1 and 3, respectively. The age of the patients ranged from 29 to 78 (mean, 62) years old. None had received irradiation or chemotherapy before surgery. Tissues used in this study were obtained from only those patients whose lymph nodes were pathologically confirmed to be involved. The most distant metastatic lymph node was selected in cases when two or more lymph nodes were involved.

Immunohistochemistry Tissue sections cut at a thickness of 4 µm were deparaffinized and rehydrated. After heat
treatment for antigen retrieval in 10 mM citrate buffer and blocking of endogenous peroxidase activity by incubation with 3% H2O2, sections were incubated with anti-human p185HER-2/neu polyclonal antibody (dilution 1:200; Dako A/S, Glostrup, Denmark) or anti-human p21CIP1/WAF1 monoclonal antibody (dilution 1:40, clone EA10; Oncogene Research Products, Darmstadt, Germany). Immunohistochemistry was performed with a Super Sensitive Multi-Link Detection Kit, HRP/DAB (BioGenex, San Ramon, CA). After counterstaining with hematoxylin, the sections were dehydrated and mounted.

Assessment of expression Assessment of immunostaining for p185HER-2/neu was based on detectable membrane labeling, and that for p21CIP1/WAF1 was based on detectable immunoreactive nuclei. Two pathologists (S. S. and H. I.), who were blinded to patient outcome, evaluated the immunohistochemical results independently. The immunohistochemical staining of p185HER-2/neu was classified positive when more than 10% of ≥500 examined tumor cells were moderately (+) or strongly (++) stained. Staining of p21CIP1/WAF1 was classified into two groups; negative when 0% to 5% of ≥500 examined tumor cells were stained, and positive when over 5% were stained, as described previously.11)

Statistical analysis Clinical and pathological characteristics were compared between groups with Fisher’s exact test for categorical variables. Associations regarding p185HER-2/neu and p21CIP1/WAF1 expression between primary and secondary tumor samples were assessed with the kappa (κ) test, and evaluated based on the following criteria: 0.93≤κ≤1.00, excellent agreement; 0.81≤κ≤0.92, very good agreement; 0.61≤κ≤0.80, good agreement; 0.41≤κ≤0.60, fair agreement; 0.21≤κ≤0.40, slight agreement; 0.01≤κ≤0.20, poor agreement; ≤0.00, no agreement.12) All analyses were two-tailed, and the criterion of significance was set at P<0.05.

RESULTS p185HER-2/neu expression was recognized as membrane-localized staining in tumor cells (Fig. 1). p21CIP1/WAF1 expression was detected in nuclei, and there was no case expressing p21CIP1/WAF1 in the cytoplasm (Fig. 2).

The positivities of p185HER-2/neu and p21CIP1/WAF1 in primary tumors were 47.4% (27/57) and 45.6% (26/57), respectively. For either molecule, no significant association was observed between staining and gender (male/female), age (younger/older), histological subtype (adenocarcinoma/squamous cell carcinoma), or disease stage (stage II/stage III and IV). These results are summarized in Table I.

As shown in Table II, the expression of p185HER-2/neu showed excellent agreement between primary tumors and lymph node metastases (57 of 57 cases showed matched staining, κ=1.000, P<0.0001, Table II). Seventeen cases positive for p185HER-2/neu were further analyzed, and the degree of p185HER-2/neu positive staining, (+) or (++), also showed very good agreement (26 of 27 cases showed matched staining, κ=0.9078, P<0.0001, Table III). The expression of p21CIP1/WAF1 showed good agreement, with 51 of 57 cases showing matched staining (κ=0.7876, P<0.0001, Table IV). When adenocarcinoma and squamous cell carcinoma cases were analyzed individually, p185HER-2/neu and p21CIP1/WAF1 expression also showed a degree of high agreement (Table V, Table VI). Twenty-

Fig. 1. Specimens of primary tumors stained with anti-p185HER-2/neu antibody. The membranes of tumor cells were clearly positive for p185HER-2/neu staining. The bar indicates 70 µm.

Fig. 2. Specimens of primary tumors stained with anti-p21CIP1/WAF1 antibody. Nuclei of tumor cells were positive for p21CIP1/WAF1 staining. The bar indicates 70 µm.
seven of 27 adenocarcinoma cases showed excellent agreement with matched staining of p185HER-2/neu (κ=1.000, P<0.0001) and 23 of 27 cases showed good agreement with matched staining of p21CIP1/WAF1 (κ=0.7033, P=0.0004). Twenty-six of 26 squamous cell carcinoma cases showed excellent agreement with matched staining of p185HER-2/neu (κ=1.000, P<0.0001) and 25 of 26 cases showed excellent agreement with matched staining of p21CIP1/WAF1 (κ=0.9394, P<0.0001). Adeno-squamous cell carcinoma and large cell carcinoma cases were too few for statistical analysis. No significant association was observed between p185HER-2/neu and p21CIP1/WAF1 expression (data not shown).

DISCUSSION

In the present study, we showed that both p185HER-2/neu and p21CIP1/WAF1 expression were highly concordant between primary tumors and corresponding lymph node metastases in NSCLC. This concordance was also observed when adenocarcinoma and squamous cell carcinoma cases were analyzed individually.

An anti-HER-2 monoclonal antibody to p185HER-2/neu-over-expressing cancers shows anti-tumor activity and is widely applied in the treatment of breast cancer.1-4 This antibody therapy has also been effective for several NSCLC cell lines,13 and clinical trials for NSCLC patients are currently underway.14,15
In contrast, overexpression of p185HER-2/neu is associated with reduced sensitivities to various anti-cancer drugs including cisplatin and paclitaxel.\(^5\)\(^-\)\(^7\)

p21\(^{CIP1/WAF1}\) is also associated with anti-cancer drug sensitivities, and cancer cells negative for p21\(^{CIP1/WAF1}\) show enhanced chemosensitivities to the DNA cross-linking agents cisplatin and nitrogen mustard, and the microtubule inhibitors paclitaxel and vincristine.\(^8\)\(^,\)\(^9\)

Therefore determination the p185\(^{HER-2/neu}\) expression is considered to be very important in not only deciding on administration of anti-HER-2 monoclonal antibody, but also predicting the sensitivity of cytotoxic agents such as paclitaxel and cisplatin. Similarly p21\(^{CIP1/WAF1}\) expression may provide additional information for this prediction. In breast cancers, overexpression of p185\(^{HER-2/neu}\) confers paclitaxel resistance, and increased p21\(^{CIP1/WAF1}\) expression is considered as one of the underlining mechanisms of this resistance.\(^10\) Increased resistance to paclitaxel was reversed by antisense inhibition of p21\(^{CIP1/WAF1}\) expression in cells overexpressing p185\(^{HER-2/neu}\). However, we failed to detect any significant association between p185\(^{HER-2/neu}\) and p21\(^{CIP1/WAF1}\) expression in this immunohistochemical study. This finding is not in accord with previous observation, but it was not considered that there is a strong correlation between p185\(^{HER-2/neu}\) and p21\(^{CIP1/WAF1}\) expression in NSCLC. Recently, cytoplasmic localization of p21\(^{CIP1/WAF1}\) was reported to be associated with p185\(^{HER-2/neu}\) overexpression through Akt-induced phosphorylation.\(^10\) Nevertheless, cytoplasmic localization of p21\(^{CIP1/WAF1}\) was not detected in this study. Although the reason is unclear, we speculate that the sensitivity of our immunostaining was not high enough to detect cytoplasmic localization of p21\(^{CIP1/WAF1}\), or that the Akt pathway may have been considerably reduced in our cohort of NSCLC tissues. Further investigation is needed to elucidate the relationship between p185\(^{HER-2/neu}\) expression and p21\(^{CIP1/WAF1}\) cytoplastic localization in NSCLC.

We showed that both p185\(^{HER-2/neu}\) and p21\(^{CIP1/WAF1}\) were expressed with a high degree of correspondence between primary tumors and metastatic nodes. Several investigators have reported that p185\(^{HER-2/neu}\) oncoprotein was equally expressed between lymph node metastases and primary tumors in patients with breast cancer.\(^17\)\(^-\)\(^20\) Taken together with our results, these observations suggest that the acquisition of p185\(^{HER-2/neu}\) overexpression may generally occur before cell dissemination from the primary tumor, and p185\(^{HER-2/neu}\) overexpression may not be a prerequisite for metastatic growth, as primary tumors with immunohistochemically undetectable p185\(^{HER-2/neu}\) can also spread to distant sites.

As regards p21\(^{CIP1/WAF1}\) expression in primary tumors and lymph node metastases, there has been only one study reported, in which a low rate of concordance of p21\(^{CIP1/WAF1}\) expression in colorectal adenocarcinomas was described.\(^21\) In the present study, however, p21\(^{CIP1/WAF1}\) expression between primary tumors and lymph node metastases showed very good agreement, and a similarly good agreement was obtained when only adenocarcinoma cases were analyzed. The reason for the discrepancy regarding p21\(^{CIP1/WAF1}\) expression between our study and that of Mckay et al. is unclear, although it may have been due to differences in characteristics between NSCLC and colorectal cancer.

For squamous cell carcinoma, we also found that both p185\(^{HER-2/neu}\) and p21\(^{CIP1/WAF1}\) were equally expressed between paired samples of primary and corresponding metastatic tumors. This has not been reported previously, and should also be examined in squamous cell carcinoma of other organs, e.g. esophageal, head and neck, and cervical carcinomas.

In summary, we showed that in NSCLC both p185\(^{HER-2/neu}\) and p21\(^{CIP1/WAF1}\) expression showed a high degree of concordance between primary and metastatic tumors, and similar results were obtained when adenocarcinoma and squamous cell carcinoma cases were analyzed individually. Both p185\(^{HER-2/neu}\) and p21\(^{CIP1/WAF1}\) are involved in the sensitivity to various anti-cancer therapies. The optimal anti-cancer drugs identified from the information obtained from the primary tumor may be expected to be effective in systemically metastasized deposits.

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