Differences in the Expression Profiles of Excision Repair Crosscomplementation Group 1, X-Ray Repair Crosscomplementation Group 1, and βIII-Tubulin Between Primary Non-small Cell Lung Cancer and Metastatic Lymph Nodes and the Significance in Mid-Term Survival

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Introduction: This study aimed to compare the expression profiles of excision repair crosscomplementation group 1 (ERCC1), x-ray repair crosscomplementation group 1 (XRCC1), and βIII-tubulin between patients with primary non-small cell lung cancer (NSCLC) and those with metastatic lymph nodes and to identify the prognostic significance of each chemotherapy resistance protein.

Materials: Those who met the inclusion criteria were patients (1) with NSCLC, (2) with metastatic lymph nodes (N1 or N2), and (3) who underwent surgical resection followed by platinum-based adjuvant chemotherapy. A total of 82 patients were included in the study. The expression profile of each protein was evaluated by immunohistochemistry and compared according to tumor location.

Results: The mean age of the patients was 57.5 ± 8.4 years. There were 30 N1 and 52 N2 patients. ERCC1 expression was upregulated in 55% and downregulated in 8% of metastatic lymph nodes, when compared with primary tumors (p < 0.05). XRCC1 was also upregulated in 56% and downregulated in 6% (p < 0.05). However, βIII-tubulin was upregulated in 12% and downregulated in 45% of patients (p < 0.05). βIII-tubulin expression in metastatic lymph nodes was greater in patients with adenocarcinoma than other cell types. Upregulation of ERCC1 in metastatic lymph nodes was a poor prognostic factor in N1 patients but not in N2 patients.

Conclusions: Significant changes in the expression profile of each protein were observed in metastatic lymph nodes. The resistance protein-guided treatment should be performed after integrative interpretation of expression profiles of each protein in both primary and metastatic sites.

Key Words: Carcinoma, Non-small cell, Thoracic surgical procedures, Chemotherapy, Adjuvant, Drug resistance, Neoplasm.

Lymph node metastasis is the most important prognostic factor in resectable non-small cell lung cancer (NSCLC). Despite complete resection of primary tumor and draining metastatic lymphatic chains, a significant number of patients suffered from recurrences at locoregional and distant sites.1–3 Adjuvant chemotherapy has been administered to patients with advanced NSCLC in an attempt to prevent distant recurrence and improve overall survival.4,5 However, the modest effect of adjuvant chemotherapy on overall survival increased the need for proper patient selection criteria and a tailored treatment regimen based on the responsiveness of cancer cells to chemotherapeutic agents. DNA repair mechanisms have recently been emphasized because several DNA repair pathways had been known to be related to the resistance to cisplatin-based chemotherapy.6 Excision repair crosscomplementation group 1 (ERCC1) and x-ray repair crosscomplementation group 1 (XRCC1) are proteins involved in the nucleotide excision repair pathway and base excision repair pathway, respectively. Several studies have reported that both proteins are related to resistance to cisplatin-based chemotherapy.7–10 The expression level of type III β-tubulin is known to be related to resistance to taxane, which affects the cancer cell by inhibiting microtubule disassembly.11,12 Recent clinical trials aimed to improve the efficacy of chemotherapy by guiding chemotherapy resistance gene expression.13 However, most of the previous studies did not consider whether the tumor tissue originated from a primary or metastatic tumor.

The aim of this study was to investigate the expression profiles of ERCC1, XRCC1, and type III β-tubulin in resected primary tumors and metastatic lymph nodes by immunohistochemistry and to identify differences between these two tumor sites and the prognostic significance of each expression profile.
PATIENTS AND METHODS

Patients

Patients who met the inclusion criteria were those (1) who underwent primary surgical resection due to NSCLC, (2) who did not receive neoadjuvant chemotherapy, (3) with nodal metastasis (pathologic stages II and IIIa), (4) who received platinum-based adjuvant chemotherapy, and (5) who had a follow-up period of more than 2 years, patients with T4 stage disease were not included in this study. From July 2000 to August 2006, 82 patients fulfilled the above criteria and were included in the study. The institutional review board of our hospital approved this study, and patient consent was waived.

Immunohistochemistry

Primary tumor and paired one metastatic lymph node were selected for immunohistochemistry. In each case, a tissue array block was made from paraffin-embedded specimens. Two-millimeter cores were taken from the specimen (donor blocks) and inserted into new paraffin blocks (tissue array blocks) using a trephine apparatus (Superbiochips Laboratories, Seoul, Republic of Korea). Immunohistochemical staining for ERCC1, XRCC1, and βIII-tubulin was performed at Superbiochips Laboratories (Seoul, Republic of Korea). Same antigen retrieval method was applied to all three markers. The tissues were pretreated using a microwave antigen retrieval procedure (700 W for 15 minutes) in 10 mM citrate buffer (pH 6.0). After incubation in 3% hydrogen peroxide for 6 minutes, immunostaining was performed using an automated immunostainer (Autoimmunostainer 360, laboratory vision, Fremont, CA). The antibodies used were as follows: anti-ERCC1 (clone 8F1, GeneTex, San Antonio, TX; dilution 1:100), anti-XRCC1 (clone 33-2-5, Laboratory vision, Fremont, CA, dilution 1:300), and anti-βIII-tubulin (clone SDL3D10, BioGenex, San Ramon, CA, dilution 1:750). A Vectastain Elite ABC kit (Vector laboratories) was used to visualize βIII-tubulin immunostaining, and an Ultravision LP kit (Laboratory Vision, Fremont, CA) was used to visualize XRCC1 and ERCC1. They were then counterstained with Mayer’s hematoxylin for 10 seconds. Evaluation of immunostaining was performed by a pathologist who was blind to the patients’ clinical information. Nuclear staining for ERCC1 and XRCC1 was interpreted as positive and cytoplasmic staining for βIII-tubulin was interpreted as positive. The intensity was graded on a scale of 0 to 3. For ERCC1 and XRCC1, endothelial cells were used as an internal control and assigned an intensity of 2. The percentage of positive tumor cells was also recorded (Figure 1).

Statistical Methods

Continuous variables are expressed as means ± SD. Nominal variables were expressed as numbers and proportions. Wilcoxon signed rank tests were used to compare the expression profiles of each resistance protein between primary and metastatic tumors. The effect of each clinical parameter on the expression level of resistance proteins was evaluated by one-way analysis of variance or Kruskal-Wallis test. The Kaplan-Meier method was used to determine overall and recurrence-free survival. Univariate analysis was performed using a log-rank test. Multivariate analysis was performed using the Cox proportional hazard model. All factors with a value of \( p < 0.10 \) by univariate analysis were included in the multivariate analysis. Statistical significance was accepted at the \( p < 0.05 \) level. Statistical analyses were performed using SPSS 13.0 software (SPSS Inc, Chicago, IL).

RESULTS

Patient Demographics

The mean age of the patients was 57.5 ± 8.4 years, and there were 52 men and 30 women. Lobectomy, bilobectomy, and pneumonectomy were performed in 61, 10, and 11 patients, respectively. The cell types comprising resected tumors were adenocarcinoma in 45, squamous cell carcinoma in 31, and others in six patients. The mean size of the resected tumors was 3.8 ± 1.5 cm, and there were 16 T1, 58 T2, and eight T3 tumors. Thirty patients showed N1 metastasis, and 52 patients showed N2 metastasis. Twenty-three patients (28%) had stage II disease, and 59 patients (72%) had stage IIIa disease (Table 1). All patients received platinum-based adjuvant chemotherapy. Cisplatin was used in 46 patients (56%), and carboplatin was used in 36 patients (44%). The combined chemotherapeutic agents were taxane in 52 (63%), vinorelbine in 19 (23%), gemcitabine in seven (9%), and etoposide in four patients (5%). The mean number of chemotherapy cycles was 3.9 ± 1.1.
Differences in Expression Profiles in Primary Tumors and Metastatic Lymph Nodes

The expression profiles of each resistance protein in primary tumor and metastatic lymph nodes were compared. ERCC1 expression was upregulated by 55% in patients with metastatic lymph nodes, when compared with those with primary tumors. However, ERCC1 expression was downregulated in only 8% of patients ($p < 0.05$; Figure 2). The pattern of XRCC1 expression was similar to that of ERCC1. XRCC1 expression was upregulated in 56% of patients and downregulated in 6% of patients (Figure 3; $p < 0.05$). However, the expression profile of βIII-tubulin was different than those of XRCC1 and ERCC1. βIII-Tubulin was upregulated in 12% of patients but downregulated in 45% of patients (Figure 4). βIII-Tubulin expression was significantly downregulated in patients with metastatic lymph nodes in comparison with those with primary tumors ($p < 0.05$).

Correlation with Other Clinical Factors

The impact of clinical factors on the expression level of each resistance protein was evaluated. Age, gender, cell type, stage, tumor size, T stage, and N stage were evaluated. The expression levels of ERCC1 and XRCC1 were not affected by any clinical factors, regardless of the tumor site (primary and metastatic; Tables 2 and 3). However, univariate analysis revealed that βIII-tubulin expression in metastatic lymph nodes was influenced by gender, cell type, and N stage. Multivariate analysis revealed that the cell type of adenocarcinoma was the only clinical factor to have a significant effect on βIII-tubulin expression in metastatic lymph nodes. Positive βIII-tubulin expression was detected in 73% of adenocarcinomas, but only 31% of other cell types showed βIII-
tubulin expression (Table 3). Clinical factors had no effect on βIII-tubulin expression in primary tumors.

**Survival**

The median follow-up period was 28.3 months (3–88 months). Twenty-three patients (28%) died during the follow-up period, and 40 patients (49%) had recurrent lung cancer. The 3- and 5-year overall survival rates were 77% and 68%, respectively. The 3-year overall survival rate was 78% in patients with stage II disease and 64% in patients with stage IIIa disease \( (p = 0.194) \). The 3- and 5-year recurrence-free survival rates were 52% and 48%, respectively. The 3-year recurrence-free survival rate was 69% in patients with stage II disease and 45% in patients with stage IIIa disease \( (p = 0.056) \). The expression level itself was not a significant prognostic factor for overall survival. The upregulation of ERCC1 expression in metastatic lymph nodes (upregulation) was a significant risk factor in N1 disease \( (p = 0.024; \text{Figure 5A}) \). However, the prognostic effect of upregulation was not identified in N2 disease (Figure 5B). The changes in XRCC1 and βIII-tubulin expression in metastatic lymph nodes did not affect the overall survival rate. We analyzed the effect of βIII-tubulin expression in the subgroup of patients who
received taxane-based chemotherapy, but we could not find any prognostic correlation. We analyzed difference in survival according to platinum regimen. The 5-year overall and recurrence-free survival was 63.1% and 46.7% in the patients with cisplatin regimen and 74.5% and 52.2% in the patients with carboplatin regimen, and there was no significant difference according to platinum regimen.

**DISCUSSION**

Complete excision of the primary tumor and draining of the lymphatic channel are mainstays of surgical treatment for NSCLC. In early-stage lung cancer, the cure rate of surgical resection ranges from 60 to 80%. However, a significant proportion of patients with NSCLC are considered to already have lymphatic or systemic metastasis at the initial presentation. Because of the early metastatic nature of NSCLC, the usual mode of treatment failure after surgical resection is distant recurrence rather than local recurrence. Therefore, the importance of systemic treatment is strongly emphasized in the treatment of resectable NSCLC. Adjuvant chemotherapy is being widely used to treat patients who undergo complete resection. Several studies reported a survival benefit of adjuvant chemotherapy. However, only a small portion of patients benefited from the adjuvant chemotherapy in those studies. To improve the effectiveness of chemotherapy, proteins resistant to specific chemotherapeutic agents have been studied. Based on the results of retrospective studies, recent studies tried to guide the chemotherapy regimen according to the expression levels of chemotherapy resistance proteins. Although a long-term result is not yet available, the early results seem promising.

Therefore, whether metastatic lesions are more aggressive than primary tumors and alter the expression profiles of various genes has not yet been determined. However, recent advances in cancer stem cell theory suggest that cancer stem cells could be the source of metastasis and drug resistance. Although cancer stem cells have not yet been detected in lung cancer, we supposed that a similar feature would be applied to NSCLC. Considering the findings of previous studies, we hypothesized that the protein expression profiles between primary tumor and metastatic site could be different. We selected ERCC1, XRCC1, and βIII-tubulin among the resistance-related proteins. ERCC1 and XRCC1, which are included in the DNA repair pathway, are related to platinum-based chemotherapy. On the other hand, βIII-tubulin is not included in the DNA repair pathway, and it is related to taxane-based chemotherapy. We compared the expression profiles of each resistance protein in primary tumors and metastatic lymph nodes.

The expression profile in this study showed clear differences between the resistance proteins. The DNA repair proteins were upregulated in metastatic lymph nodes, whereas βIII-tubulin was downregulated. Whether the metastatic lesions have the same gene expression profiles as primary tumors remain a subject of debate. Many studies reported conflicting results. However, recent advances of cancer stem cell theory raised another issue in chemotherapy resistance. Cancer stem cells are first found as engrafted cells when injected into immune-deficient mice, which have the self-renewing and metastatic potential throughout the entire cancer cell population. This theory can partially explain the heterogeneity of tumor cell populations and partially explain the drug resistance in metastatic lesions. Current studies suggested that cancer stem cells could be a significant cause of drug resistance. Cancer stem cells exhibit little dividing activity and many cellular mechanisms to evading chemotherapy agents. One of the main mechanisms of this resistance is known to be related to the capacity for DNA repair. Until recently, the presence of cancer stem cells in lung cancer was unclear. Furthermore, we cannot say that the result of our study represents the metastasis of selected clones of cancer stem cells. However, we could identify that the metastatic clones of NSCLC in this study showed increased expression levels of DNA repair proteins.
Generally, it has been known that increased βIII-tubulin expression is related to high-grade malignancy and cell type. Katsetos et al. analyzed the expression levels of βIII-tubulin in lung cancer, and they reported that the expression level was higher in neuroendocrine tumors but lower in other types of NSCLC. They also reported that βIII-tubulin expression in adenocarcinoma is highly correlated to lymph node metastasis. However, their report could not show any statistical significance due to the small number of samples. Our study also showed similar results. There was no difference in βIII-tubulin expression at the primary tumor site between adenocarcinoma and other cell types. However, the βIII-tubulin expression in metastatic lymph nodes was clearly higher in adenocarcinoma. The role of βIII-tubulin in NSCLC metastasis is not yet known. Thus, further studies are necessary to determine the role of βIII-tubulin in lymphatic metastasis.

Although we tried to identify the prognostic significance of three proteins that are known to be related to drug resistance, the absolute expression level itself was not related to the prognosis. The only significant risk factor was upregulation of ERCC1 expression in the early stages of metastasis (N1). We believe that there may be two possible explanations for this finding. The first possible explanation is that all of the patients in this study received cisplatin-based chemotherapy. The prognostic correlation between ERCC1 and cisplatin-based chemotherapy had already been reported by many studies. However, the significance of XRCC1 in cisplatin-based chemotherapy was not well established until now and that of βIII-tubulin remains unclear. Therefore, ERCC1 could be the only significant risk factor. The second explanation concerns the limited prognostic value in the early stages of metastasis. We hypothesized that upregulation in metastatic lymph nodes shows that more aggressive and chemotherapy-resistant clones metastasized to adjacent lymph nodes. Therefore, it could be possible that the prognosis of patients with N1 disease with no or downregulated expression was good. However, N2 metastasis represents a higher chance and burden of systemic metastasis. Although the degree of resistance might be different, the benefit of chemotherapy would not be great.

In this study, we compared the expression profiles of resistance proteins between primary tumor and metastatic lymph nodes in patients who underwent surgical resection and adjuvant chemotherapy due to NSCLC. DNA repair proteins, including ERCC1 and XRCC1 were upregulated in metastatic tumors, when compared with primary tumors; however, βIII-tubulin was downregulated in metastatic tumors. Upregulation of ERCC1 in metastatic lymph nodes was a significant risk factor for overall survival in N1 metastasis but not in N2 metastasis. Therefore, we believe it is necessary to decide which site, primary or metastatic, should be the reference site of protein expression in prospectively designed clinical trials evaluating chemotherapy resistance.

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REFERENCES