Increase in the Frequency of p16INK4 Gene Inactivation by Hypermethylation in Lung Cancer during the Process of Metastasis and Its Relation to the Status of p53

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ABSTRACT

The p16INK4 gene, which is a tumor suppressor gene, is frequently altered in lung cancers. Hypermethylation of the promoter region of the p16INK4 gene seems to be the major mechanism through which p16INK4 becomes inactivated. Hypermethylation of the p16INK4 gene was reported to occur at an early stage in lung cancer. To determine whether the change in p16INK4 methylation status occurs at the late stage in the progression of primary lung cancers, we analyzed the primary and metastatic tumor tissues and normal lung samples from 29 cases of advanced lung cancer with distant metastasis. In each tissue sample, we analyzed the p16INK4 and p15INK4b genes for mutations and the methylation status of both genes using PCR-single strand conformation polymorphism, direct sequencing, and methylation-specific PCR analysis. We also analyzed a subset of the samples for p16INK4 protein expression. Genetic mutations in the coding region of the p16INK4 and p15INK4b genes were not found in any of the examined specimens. The promoter region of the p16INK4 gene was hypermethylated in the tumor samples of the primary or metastatic site of 37.0% (10 of 27) of the subjects. The promoter region of the p16INK4 gene was hypermethylated at both the primary and metastatic sites in two of the 10 cases and at only the metastatic site in 8 cases. By immunohistochemical analysis, we confirmed the presence of p16INK4 protein at the primary site of all cases in which the promoter region of the p16INK4 gene was hypermethylated at only the metastatic site. Interestingly, all 8 cases with a hypermethylated p16INK4 promoter region, at only the metastatic site, did not have p53 mutation. The results of this study indicate that tumor cells in which the p16INK4 gene has been inactivated by hypermethylation of the promoter region could have an advantage in progression and metastasis in non-small cell lung cancers, especially in the tumors with normal p53, and that the frequency of p16INK4 gene inactivation by hypermethylation could vary in clinical course.

INTRODUCTION

The development of human cancers including lung cancers is associated with an accumulation of genetic alterations of tumor suppressor genes. Several tumor suppressor genes or candidates, including p53 (1–3), p16INK4, p15INK4b (4–7), and FHIT (8, 9), have been reported to be mutated or deleted in lung cancer. CDKIs3 control the progression through the cell cycle by binding to CDK, thereby inhibiting its activity, and by rendering the retinoblastoma protein (RB) inactive (10, 11). Among the genes that encode CDKIs, p16INK4 is genetically and epigenetically altered in many cancer cell lines (4, 12–14). Homozygous deletion of this gene has been detected in a high percentage (28–71%) of human lung cancer cell lines (4–7). Homozygous deletion or point mutations of p16INK4 are not frequently observed among primary lung cancers but are observed among metastatic and advanced NSCLCs (5, 6, 15).

An alternative mechanism of p16INK4 inactivation is aberrant methylation of the CpG island promoters, and this is common in a number of human cancers including esophageal cancer (38%; Ref. 16), breast cancer (31%), and colon cancer (40%; Ref. 17). Aberrant methylation of normally unmethylated CpG islands is associated with transcriptional inactivation and loss of expression of tumor suppressor genes in human cancers. Aberrant methylation of the p16INK4 gene is observed frequently in NSCLCs; in 36–64% of cell lines (18–20) and 16–53% of primary tumors (14, 15, 19, 21). Hypermethylation is thought to be the major mechanism through which p16INK4 becomes inactivated in primary lung cancers. p16INK4 hypermethylation was reported to be frequently detected in premalignant lesions (22). However, it is still unknown whether the methylation status of the p16INK4 gene status changes during the progression of lung carcinoma.

The p15INK4b gene is located 25 kb from the p16INK4 gene on chromosome 9p21. Homozygous deletion is the most frequently observed alteration of this gene. Deletion of the

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3 The abbreviations used are: CDKI, cyclin-dependent kinase inhibitor; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; SSCP, single strand conformation polymorphism; MSP, methylation-specific PCR.
**Table 1**  Patient characteristics and results of p16\(^{INK4a}\) methylation and p53 mutation

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* Methyl, methylated; un, unmethylated; adeno, adenocarcinoma; sq, squamous cell carcinoma; ad-sq, adeno-squamous cell carcinoma; CT, chemotherapy; Effect, response to chemotherapy; Prim, Primary; Meta, Metastasis; mut, mutation; PR, partial response; MR, minor response; NC, no change; PD, progressive disease; NE, not evaluated; ND, not done.

\(p15^{INK4b}\) gene frequently occurs concurrently with deletion of \(p16^{INK4a}\) (23). In rare cases, the \(p15^{INK4b}\) gene is deleted without any alteration of the \(p16^{INK4a}\) gene. Mutation or hypermethylation of the \(p15^{INK4b}\) gene is rare in most human tumors (19, 24).

In this study, we analyzed the genetic and methylation status of the \(p16^{INK4a}\) and \(p15^{INK4b}\) genes at the primary and metastatic sites of lung cancer, as well as at a normal lung site. In addition, we analyzed the protein expression of \(p16^{INK4a}\) in a subset of the lung cancer samples. We also compared the genetic status of these genes with the status of the \(p53\) gene, which is frequently mutated in cancers and is involved in the \(G_1\) checkpoint to determine whether these genes become inactivated during the progression of primary lung cancer.

**MATERIALS AND METHODS**

**Tissue Samples.** This study included 29 subjects with primary lung cancer with distant metastasis who had been admitted at the Fourth Department of Internal Medicine at Nippon Medical School Main Hospital. All of the subjects in this study had metastatic cancer at the time of autopsy. We obtained tissue samples from the primary site, any distant metastatic site excluding lymph nodes, and a normal lung site from each subject. Each sample was frozen at −80°C. The histological type of lung cancer of the 29 cases included 6 SCLCs, 13 adenocarcinomas, 8 squamous cell carcinomas, one large cell carcinoma, and one adenosquamous cell carcinoma. The characteristics of the 29 cases are shown in Table 1. Genomic DNA was extracted from the tumor specimens and normal samples by proteinase K treatment and phenol chloroform extraction using standard protocols (7, 25, 26).

**PCR-SSCP Analysis.** Exons 1 and 2 of each of the \(p16^{INK4a}\) and \(p15^{INK4b}\) genes in the DNA samples obtained from the primary site, metastatic site, and normal site of each patient were examined for the presence of a mutation by PCR-SSCP analysis. Each exon was amplified using the PCR primers, the sequences of which are shown in Table 2. PCR was performed using the Gene Amp XL and XL Buffer kit (Perkin-Elmer Corp., Foster City, CA) as follows: denaturation at 94°C for 40 s, annealing at 55°C for 30 s, and extension at 68°C for 90 s for 40 cycles, with a final extension at 68°C for 8 min. The PCR reaction mixture consisted of 1× XL buffer, 1100 μM Mg(OA2), 200 μM deoxynucleotide triphosphate, 0.3 μM of each primer, 10 μCi of \(^{32}\)P-dCTP, 0.5 units of \(Tth\) DNA polymerase, and 25 ng of genomic DNA. The \(^{32}\)P-labeled PCR products were denatured, cooled on ice, loaded on neutral 6% polyacrylamide gel with and without 5% (vol/vol) glycerol, electrophoresed, dried, and exposed to X-ray film at −80°C overnight (7, 25, 26).

**DNA Sequence Analysis.** DNA sequence analysis of the aberrant bands was performed as described previously.
(7, 25, 26). Briefly, the aberrant bands were excised and further amplified by PCR using sequencing primers with the M13 sequence (TGTTAAACGACGCGCCAGT) added to the appropriate PCR primers. The PCR product was purified and sequenced using a fluorescent automated sequencer (Perkin-Elmer Corp./Applied Biosystems, Inc., Foster City, CA).

**Methylation Analysis.** The methylation status of the promoter regions of the *p16* and *p15* genes was analyzed by MSP (27) using the CpG DNA Modification kit (Intergen Company, Purchase, NY) and CpG WIZ Amplification kit (Intergen Company). Each DNA sample was subject to bisulfite modification. One μg of DNA was placed in 100 μl of water and denatured by adding 7.0 μl of 3 M NaOH for 10 min at 37°C. To each denatured DNA solution was added 550 μl of freshly prepared sodium bisulfite mixture (Intergen Company), and these solutions were incubated at 50°C for 16–20 h. In the bisulfite modification, all unmethylated cytosines are deaminated and converted to uracils, whereas the 5-methylcytosines remain unaltered. The DNA samples were then purified by ethanol precipitation and resuspended in 25–50 μl of TE (10 mM Tris/0.1 mM EDTA, pH 7.5). PCR amplification was performed with methylation-specific primers (Intergen Company) that had been designed to distinguish methylated from unmethylated DNA. The PCR reaction mixture consisted of 1× Universal PCR buffer, 250 μM deoxynucleotide triphosphate, 0.2 μM of each primer, 1.25 units of AmpliTaq Gold, and 2 ng of template DNA. The PCR reaction mixture consisted of 1× Universal PCR buffer, 250 μM deoxynucleotide triphosphate, 0.2 μM of each primer, 1.25 units of AmpliTaq Gold, and 2 ng of template DNA. The PCR product was purified and sequenced using a fluorescent automated sequencer (Perkin-Elmer Corp./Applied Biosystems, Inc., Foster City, CA).

**Immunohistochemistry.** Immunohistochemical analysis for *p16* expression was performed on paraffin sections of tumors using the monoclonal antibody F-12 (JgG2a mouse monoclonal; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used. Three-μm-thick paraffin sections of the lesions were mounted on silan-coated slides, dewaxed, rehydrated, incubated for 30 min with 0.3% hydrogen peroxide, and boiled in 10 mM citrate buffer (pH 6.0) in a microwave oven for 2–10 min. The slides were incubated with mouse monoclonal antibody to 1:50 dilution at 4°C overnight. Biotin-conjugated secondary antibody was added at 1:50 dilution for 1 h at room temperature. The slides were then incubated with biotinylated goat-anti-mouse IgG (1:200; Sigma Chemical Co., St. Louis, MO) and streptavidin-horseradish peroxidase (1:500; Vector Laboratories, Inc., Burlingame, CA) for 30 min each. Substrate chromagen solution was then applied to the slides for 5 min. The slides were then rinsed and counterstained with hematoxylin. Immunohistochemical analysis of the *p16* promoter region was performed on specimens obtained from the primary and metastatic sites of 27 subjects to determine the methylation status of the *p16* promoter region at each site. The methylation status of the *p16* promoter region was scored according to the percentage of tumor cells that were stained, as follows: +++, markedly positive (>50% of tumor cells were intensely stained); +, mildly positive (10–50% of tumor cells were intensely stained); and −, negative (<10% of tumor cells were intensely stained).

**RESULTS**

We analyzed exon 1 and exon 2 of the *p16* and *p15* genes for the presence of a mutation by PCR-SSCP analysis using intron primers in specimens obtained from the primary, metastatic site, and normal lung site of 29 subjects who had primary lung cancer with distant metastasis. PCR-SSCP analysis of the *p15* gene revealed aberrant bands in the primary site, metastatic site, and normal lung site of one small cell carcinoma case, case 28. DNA sequence analysis of the aberrant band in the primary and metastatic sites of case 28 revealed a C-to-G substitution in the noncoding region of intron 1 of the *p15* gene downstream of exon 1, which seemed to be a polymorphism (Fig. 1). There were no other detectable mutations of the *p16* or *p15* gene in the tumor samples of the primary and metastatic sites and normal samples by PCR-SSCP analysis in this series of lung cancer cases.

MSP was performed on specimens obtained from the primary, metastatic, and normal lung sites of 27 subjects to determine the methylation status of the promoter region of the *p16* gene and that of the *p15* gene at each site. The methylation band of the *p16* gene was observed in samples obtained from the primary and/or metastatic site of 10 (37.0%) of the 27 subjects (Fig. 2). The DNA sequence of the methylated bands was confirmed (Fig. 2). Nine (42.8%) of the 21 NSCLC cases and 1 (16.7%) of the 6 SCLC cases (Table 1; Fig. 2) showed hypermethylation of the *p16* gene at the primary and/or metastatic site. Methylation analysis of the *p16* gene in each tumor sample revealed an unmethylated band attributable to contamination of normal cells. The nine NSCLC cases with aberrant methylation of the *p16* gene consisted of 5 squamous cell carcinoma cases (5 of 8; 62.5%), 3 adenocarcinoma cases (3 of 11; 27.3%), and 1 adeno-squamous carcinoma case (1 of 1). Moreover, the methylation status in the primary site and metastatic site of each patient was not always identical. Eight cases had a hypermethylated *p16* promoter region at only the metastatic site. Of these cases with aberrant methylation at only the metastatic site, one case (case 14) did not receive chemotherapy. In this patient, hypermethylation of the *p16* promoter region had occurred during the process of metastasis. Of the other 7 cases in which hypermethylation was seen only at the metastatic site, chemotherapy was started after metastasis had occurred in three cases (cases 13, 22, and 27). Of these three cases, 11 cases had primary lung cancer with distant metastasis.
cases, chemotherapy was effective on the primary and metastatic site tumors of cases 13 and 27, and it was not effective on the tumors of case 22. Metastasis in the other 4 cases occurred after chemotherapy was started, and chemotherapy was not effective in these cases. Aberrant methylation of the \textit{p15 INK4b} gene was not detected by MSP analysis in any of the specimens. None of the normal lung specimens had hypermethylation of the promoter region of the \textit{p16 INK4a} or \textit{p15 INK4b} gene.

We performed immunohistochemical analysis for \textit{p16 INK4a} protein on the tumors of cases that had a hypermethylated \textit{p16 INK4a} promoter region at only the metastatic site (Fig. 3). The primary site of 6 of the 8 cases was available for staining. The
primary sites of all of these cases, which did not have a hypermethylated p16INK4 promoter, were positive for p16INK4 protein. The nuclei of these primary lung cancer cells were stained for p16INK4, but the staining for p16INK4 protein was heterogeneous within each tumor (Fig. 3).

We reported previously that 31% of these human lung cancer cases had a mutation in p53, which was present at both the primary and metastatic sites (Ref. 3; Table 1). Interestingly, hypermethylation of the p16INK4 gene was present in only one (11.1%) of the 9 cases with p53 mutation (case 17), whereas 9 (50%) of the 18 cases that did not have p53 mutation had hypermethylation of the p16INK4 gene at the primary and/or metastatic site (P = 0.0912 by two-sided Fisher’s exact test). All 8 cases with a hypermethylated p16INK4 promoter region at only the metastatic site did not have p53 mutation.

**DISCUSSION**

The p16INK4 gene is a candidate tumor suppressor gene in human lung cancer. Homozygous deletion and hypermethylation have been reported to be the major mechanisms of p16INK4 inactivation in lung cancer. An association between genetic or epigenetic alteration of p16INK4 on the one hand and respiratory carcinogenesis and tumor progression on the other has been described. Nakagawa et al. (5) and Okamoto et al. (6) reported that deletion or mutation of the p16INK4 gene is frequently observed among cases of NSCLC in the advanced stage. Belinsky et al. (22) proposed that aberrant methylation of the p16INK4 gene is an early event in lung carcinogenesis because aberrant methylation was frequently detected in premalignant lesions. In our study of lung cancer with distant metastasis, aberrant methylation of the p16INK4 gene at the primary and/or metastatic site...
was also observed frequently (37.0%). The methylation status of the p16INK4 gene at the primary site and metastatic site was not always identical, and the frequency of the p16INK4 gene inactivation at the metastatic site was higher than that at the primary site (2 cases at the primary site and 10 cases at the metastatic site) in our study. To reinforce the results of MSP analysis, we performed immunohistochemical analysis for p16INK4 protein. We confirmed the presence of p16INK4 protein at the primary site of all 6 cases in whom the p16INK4 gene hypermethylation was present at only the metastatic site. The presence of a p53 mutation at the primary site and metastatic site of each patient was identical (3), and p53 mutation seemed to have occurred before the metastatic process. Tumors with the hypermethylation of the p16INK4 promoter region may appear or proliferate at a later stage in the progression of lung cancer (Table 1). In the present study, 2 of 27 cases had the p16INK4 hypermethylation in the primary tumor; therefore, the p16INK4 hypermethylation can occur in the early stage of lung cancer. The relationship between reduced expression of p16INK4 and stage of disease has also been reported at the protein level. Brambilla et al. (28) observed loss of expression of the p16INK4 gene in invasive lung lesions. Gorgoulis et al. (29) reported frequent loss of p16INK4 expression in stage 1 and stage 2 lung cancers. Other studies have reported that there is no correlation between loss of expression of p16INK4 and the clinical stage of cancer (30, 31). These results suggest that loss of p16INK4 expression at the protein level might occur at an early stage in many cases. The studies of Nakagawa et al. (5) and Okamoto et al. (6) indicate that genetic alteration of the p16INK4 gene can occur at the late stage. In general, genetic alteration of the p16INK4 gene is detected more frequently in cell lines than in primary tumors. Genetic alteration of the gene may occur during the progression of lung cancer. Our results suggested that epigenetic alteration of the gene can also occur at the late stage, which is not during the evolution of an early cancer from precursor lesions. The tumors with the alteration can preferentially proliferate. Nuovo et al. (32) also reported that MSP-in situ hybridization revealed that p16INK4 gene hypermethylation occurs heterogeneously within early cervical tumor cell populations. The frequency of p16INK4 protein alteration is higher than the frequency of the p16INK4 gene alteration (7). That the results of studies performed at the genetic and epigenetic level differ from the results of studies performed at the protein level might indicate the existence of posttranscriptional and/or posttranslational regulation of p16INK4 as well as other CDKIs (7). Sanchez-Cespedes et al. (33) reported that although loss of 9p21 occurs early and frequently in NSCLCs, it appeared that complete inactivation of p16INK4 occurred later in the progression of cancer in vivo.

Of the 8 cases with p16INK4 hypermethylation at only the metastatic site in the present study, chemotherapy was not performed in case 14. The tumors of 2 cases (cases 13 and 27) were initially sensitive to chemotherapy, and the tumors of the remaining 5 cases were resistant to chemotherapy. In the case that did not receive chemotherapy and in the 5 cases with chemotherapy-resistant tumors, chemotherapy may not have influenced alteration of the promoter region of the p16INK4 gene in these cases.

Nine of the 18 tumors that did not have a p53 mutation had the p16INK4 gene hypermethylation at the primary and/or metastatic site, whereas only one of the 9 tumors with a mutation in p53 had the p16INK4 gene hypermethylation. Of the 8 cases with the p16INK4 gene hypermethylation at only the metastatic site, none had a p53 mutation. Previous studies have described the relationship between the inactivation of the p16INK4 and p53 genes in human cancers. Those have yielded conflicting results. In protein analyses, Vonlanthen et al. (34) reported that loss of p16INK4 expression was actually correlated with p53 overexpression. Geradts et al. (30) reported that there was no relationship between p16INK4 and p53 expression. There have only been a few genetic studies on the relationship between alterations of the p16INK4 gene and p53 gene in lung cancer because of the low frequency of genetic alteration of the p16INK4 gene. A study reported that the alterations of the INK4A/ARF locus frequently coexist with p53 mutations in NSCLCs (33), and that an inverse relationship between genetic alteration of p14ARF and genetic alteration of p53 does not exist. There are differences in the details of case selection and analysis methods in detail among the present and previous studies. No previous report has studied stage IV lung cancers. The interpretation of our results on the relationship between the p16INK4 gene inactivation and p53 status is that cells with the p16INK4 gene hypermethylation might have an advantage during the process of metastasis in tumors with normal p53 in lung cancer.

Ablation of p16INK4 hypermethylation is rare in most human tumors including lung cancer (19). Ablation of p15INK4 was not detected in the present study. This gene does not seem to be involved in the carcinogenesis and progression of lung cancer but rather seems to be involved exclusively in gliomas, non-Hodgkin’s lymphoma, and leukemias, as reported previously (18, 35, 36).

In summary, the p16INK4 hypermethylation was observed frequently among NSCLCs. Because there were cases in which the promoter region of the p16INK4 gene was methylated only at the metastatic site, epigenetic alteration of the gene can occur at the late stage, after the evolution of an early cancer from precursor lesions, and tumor cells that have a hypermethylated p16INK4 gene may proliferate in the late stage of the progression of NSCLC, especially during the metastatic process. Tumors with aberrant methylation of the p16INK4 gene might have stronger metastatic potential. Examining the methylation status of the p16INK4 gene might be useful for predicting the prognosis of patients and for treatment decisions after surgery in lung cancer. Because the p16INK4 hypermethylation was observed frequently in tumors with normal p53, the tumor cells with the inactivated p16INK4 may preferentially proliferate during the process of metastasis in tumors with normal p53.

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Increase in the Frequency of $p16^{\text{INK4}}$ Gene Inactivation by Hypermethylation in Lung Cancer during the Process of Metastasis and Its Relation to the Status of p53

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