Increase in the Frequency of p16\(^{INK4}\) Gene Inactivation by Hypermethylation in Lung Cancer during the Process of Metastasis and Its Relation to the Status of p53\(^1\)

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ABSTRACT

The p16\(^{INK4}\) gene, which is a tumor suppressor gene, is frequently altered in lung cancers. Hypermethylation of the promoter region of the p16\(^{INK4}\) gene seems to be the major mechanism through which p16\(^{INK4}\) becomes inactivated. Hypermethylation of the p16\(^{INK4}\) gene was reported to occur at an early stage in lung cancer. To determine whether the change in p16\(^{INK4}\) 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 p16\(^{INK4}\) and p15\(^{INK4b}\) 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 p16\(^{INK4}\) protein expression. Genetic mutations in the coding region of the p16\(^{INK4}\) and p15\(^{INK4b}\) genes were not found in any of the examined specimens. The promoter region of the p16\(^{INK4}\) 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 p16\(^{INK4}\) 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 p16\(^{INK4}\) protein at the primary site of all cases in which the promoter region of the p16\(^{INK4}\) gene was hypermethylated at only the metastatic site. Interestingly, all 8 cases with a hypermethylated p16\(^{INK4}\) promoter region, at only the metastatic site, did not have p53 mutation. The results of this study indicate that tumor cells in which the p16\(^{INK4}\) 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 p16\(^{INK4}\) 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), p16\(^{INK4}\), p15\(^{INK4b}\) (4–7), and FHIT (8, 9), have been reported to be mutated or deleted in lung cancer. CDKIs\(^3\) 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, p16\(^{INK4}\) 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 p16\(^{INK4}\) are not frequently observed among primary lung cancers but are observed among metastatic and advanced NSCLCs (5, 6, 15).

An alternative mechanism of p16\(^{INK4}\) 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 p16\(^{INK4}\) 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 p16\(^{INK4}\) becomes inactivated in primary lung cancers. p16\(^{INK4}\) hypermethylation was reported to be frequently detected in premalignant lesions (22). However, it is still unknown whether the methylation status of the p16\(^{INK4}\) gene status changes during the progression of lung carcinoma.

The p15\(^{INK4b}\) gene is located 25 kb from the p16\(^{INK4}\) 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.
p15INK4b gene frequently occurs concurrently with deletion of p16INK4a (23). In rare cases, the p15INK4b gene is deleted without any alteration of the p16INK4a gene. Mutation or hypermethylation of the p15INK4b gene is rare in most human tumors (19, 24).

In this study, we analyzed the genetic and methylation status of the p16INK4a and p15INK4b 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 p16INK4 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 G1 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 p16INK4a and p15INK4b 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 [32P]dCTP, 0.5 units of γTth DNA polymerase, and 25 ng of genomic DNA. The 32P-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.

### Table 1 Patient characteristics and results of p16INK4 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.
briefly, the aberrant bands were excised and further amplified by PCR using sequencing primers with the M13 sequence (TGTAAACGACGGCCAGT) 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 *p16INK4* and *p15INK4b* 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 1X 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 in a final volume of 25 μl. The PCR was performed as follows: denaturation at 95°C for 12 min, then denaturation at 95°C for 45 s, annealing at 60°C for 45 s, and extension at 72°C for 60 s for 35 cycles. Ten μl of each allele-specific PCR sample was mixed with 2 μl of loading dye. It was loaded on a 2% agarose gel and stained with ethidium bromide.

**Immunohistochemistry.** Immunohistochemical analysis for *p16INK4* expression was performed on paraffin sections of tumors. The monoclonal antibody F-12 (IgG2a 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 at 1:50 dilution at 4°C overnight. Biotin-conjugated secondary antibody was added at 1:50 dilution for 1 h at room temperature. We then used diaminobenzidine for color development and hematoxylin as the counterstain. A section was considered to be immunohistochemically positive for *p16INK4* if tumor nuclei were stained or without cytoplasmic staining. The staining pattern 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 *p16INK4* and *p15INK4b* 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 *p15INK4b* 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 *p15INK4b* gene downstream of exon 1, which seemed to be a polymorphism (Fig. 1). There were no other detectable mutations of the *p16INK4* or *p15INK4b* 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 *p16INK4* gene and that of the *p15INK4b* gene at each site. The methylated band of the *p16INK4* 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 were confirmed (Fig. 2). Nine (42.8%) of the 21 NSCLC and 1 (16.7%) of the 6 SCLC cases (Table 1; Fig. 2) showed hypermethylation of the *p16INK4* gene at the primary and/or metastatic site. Methylation analysis of the *p16INK4* gene in each tumor sample revealed an unmethylated band attributable to contamination of normal cells. The nine NSCLC cases with aberrant methylation of the *p16INK4* 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 *p16INK4* 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 *p16INK4* 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, 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 \( p15^{INK4b} \) 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 \( p16^{INK4} \) or \( p15^{INK4b} \) gene.

We performed immunohistochemical analysis for \( p16^{INK4} \) protein on the tumors of cases that had a hypermethylated \( p16^{INK4} \) promoter region at only the metastatic site (Fig. 3). The primary site of 6 of the 8 cases was available for staining. The

Fig. 1 DNA sequence analysis of the aberrant bands of the \( p15^{INK4b} \) gene revealed a C-to-G nucleotide substitution in intron 1 of \( p15^{INK4b} \) downstream of exon 1 in case 28.

Fig. 2 A, methylation analysis of the \( p16^{INK4} \) gene by MSP. The PCR product (151 bp for U, 150 bp for M) indicates whether the unmethylated (U) or methylated (M) the \( p16^{INK4} \) gene allele is present in that sample. The unmethylated allele was present in all of the samples because of contamination of normal tissue. In cases 13 and 16, only the metastatic site had the methylated \( p16^{INK4} \) gene allele. Both the primary and metastatic sites of case 8 had the methylated \( p16^{INK4} \) gene allele. P, primary site; M, metastatic site; N, normal site. B, the DNA sequence of the unmethylated band in case 8. C, the DNA sequence of the methylated band in case 8.
primary sites of all of these cases, which did not have a hypermethylated p16\textsuperscript{INK4} promoter, were positive for p16\textsuperscript{INK4} protein. The nuclei of these primary lung cancer cells were stained for p16\textsuperscript{INK4}, but the staining for p16\textsuperscript{INK4} 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 p16\textsuperscript{INK4} 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 p16\textsuperscript{INK4} gene at the primary and/or metastatic site ($P = 0.0912$ by two-sided Fisher’s exact test). All 8 cases with a hypermethylated p16\textsuperscript{INK4} promoter region at only the metastatic site did not have p53 mutation.

**DISCUSSION**

The p16\textsuperscript{INK4} gene is a candidate tumor suppressor gene in human lung cancer. Homozygous deletion and hypermethylation have been reported to be the major mechanisms of p16\textsuperscript{INK4} inactivation in lung cancer. An association between genetic or epigenetic alteration of p16\textsuperscript{INK4} 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 p16\textsuperscript{INK4} gene is frequently observed among cases of NSCLC in the advanced stage. Belinsky et al. (22) proposed that aberrant methylation of the p16\textsuperscript{INK4} 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 p16\textsuperscript{INK4} gene at the primary and/or metastatic site

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*Fig. 3* Immunohistochemistry of p16\textsuperscript{INK4} of the cases that had a hypermethylated p16\textsuperscript{INK4} promoter region at only the metastatic site. A and B, p16\textsuperscript{INK4} markedly positive immunostaining in the primary sites of a adenocarcinoma (case 13; A) and a squamous cell carcinoma (case 16; B) and strong p16\textsuperscript{INK4} nuclear reactivity in most tumor cells. C, p16\textsuperscript{INK4} mildly positive staining in the primary site of an adeno-squamous cell carcinoma (case 14) and strong p16\textsuperscript{INK4} nuclear reactivity in some tumor cells. D and E, p16\textsuperscript{INK4} staining in a small cell carcinoma (case 27). The nuclei of primary lung cancer cells were mildly stained for p16\textsuperscript{INK4} (D), but the cancer cells of the metastatic site were negative for p16\textsuperscript{INK4} (E).
was also observed frequently (37.0%). The methylation status of the p16\textsuperscript{INK4}\textsubscript{a} gene at the primary site and metastatic site was not always identical, and the frequency of the p16\textsuperscript{INK4}\textsubscript{a} 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 p16\textsuperscript{INK4}\textsubscript{a} protein. We confirmed the presence of p16\textsuperscript{INK4}\textsubscript{a} protein at the primary site of all 6 cases in whom the p16\textsuperscript{INK4}\textsubscript{a} 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 p16\textsuperscript{INK4}\textsubscript{a} 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 p16\textsuperscript{INK4}\textsubscript{a} hypermethylation in the primary tumor; therefore, the p16\textsuperscript{INK4}\textsubscript{a} hypermethylation can occur in the early stage of lung cancer. The relationship between reduced expression of p16\textsuperscript{INK4}\textsubscript{a} and stage of disease has also been reported at the protein level. Brambilla et al. (28) observed loss of expression of the p16\textsuperscript{INK4}\textsubscript{a} gene in preinvasive lung lesions. Gorgoulis et al. (29) reported frequent loss of p16\textsuperscript{INK4}\textsubscript{a} expression in stage 1 and stage 2 lung cancers. Other studies have reported that there is no correlation between loss of expression of p16\textsuperscript{INK4}\textsubscript{a} and the clinical stage of cancer (30, 31). These results suggest that loss of p16\textsuperscript{INK4}\textsubscript{a} 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 p16\textsuperscript{INK4}\textsubscript{a} gene can occur at the late stage. In general, genetic alteration of the p16\textsuperscript{INK4}\textsubscript{a} 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\textit{in situ} hybridization revealed that p16\textsuperscript{INK4}\textsubscript{a} gene hypermethylation occurs heterogeneously within early cervical tumor cell populations. The frequency of p16\textsuperscript{INK4}\textsubscript{a} protein alteration is higher than the frequency of the p16\textsuperscript{INK4}\textsubscript{a} 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 p16\textsuperscript{INK4}\textsubscript{a} 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 the p16\textsuperscript{INK4}\textsubscript{a} gene occurred later in the progression of cancer in vivo.

Of the 8 cases with p16\textsuperscript{INK4}\textsubscript{a} 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 p16\textsuperscript{INK4}\textsubscript{a} gene in these cases.

Nine of the 18 tumors that did not have a p53 mutation had the p16\textsuperscript{INK4}\textsubscript{a} gene hypermethylation at the primary and/or metastatic site, whereas only one of the 9 tumors with a mutation in p53 had the p16\textsuperscript{INK4}\textsubscript{a} gene hypermethylation. Of the 8 cases with the p16\textsuperscript{INK4}\textsubscript{a} gene hypermethylation at only the metastatic site, none had a p53 mutation. Previous studies have described the relationship between the inactivation of the p16\textsuperscript{INK4}\textsubscript{a} and p53 genes in human cancers. Those have yielded conflicting results. In protein analyses, Vonlanthen et al. (34) reported that loss of p16\textsuperscript{INK4}\textsubscript{a} expression was actually correlated with p53 overexpression. Geradts et al. (30) reported that there was no relationship between p16\textsuperscript{INK4}\textsubscript{a} and p53 expression. There have only been a few genetic studies on the relationship between alterations of the p16\textsuperscript{INK4}\textsubscript{a} gene and p53 gene in lung cancer because of the low frequency of genetic alteration of the p16\textsuperscript{INK4}\textsubscript{a} 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 p14\textsuperscript{ARF} 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 p16\textsuperscript{INK4}\textsubscript{a} gene inactivation and p53 status is that cells with the p16\textsuperscript{INK4}\textsubscript{a} gene hypermethylation might have an advantage during the process of metastasis in tumors with normal p53 in lung cancer.

Aberrant methylation of p15\textsuperscript{INK4b} is rare in most human tumors including lung cancer (19). Aberrant methylation of p15\textsuperscript{INK4b} 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 p16\textsuperscript{INK4}\textsubscript{a} hypermethylation was observed frequently among NSCLCs. Because there were cases in which the promoter region of the p16\textsuperscript{INK4}\textsubscript{a} 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 p16\textsuperscript{INK4}\textsubscript{a} gene may proliferate in the late stage of the progression of NSCLC, especially during the metastatic process. Tumors with aberrant methylation of the p16\textsuperscript{INK4}\textsubscript{a} gene might have stronger metastatic potential. Examining the methylation status of the p16\textsuperscript{INK4}\textsubscript{a} gene might be useful for predicting the prognosis of patients and for treatment decisions after surgery in lung cancer. Because the p16\textsuperscript{INK4}\textsubscript{a} hypermethylation was observed frequently in tumors with normal p53, the tumors with the inactivated p16\textsuperscript{INK4}\textsubscript{a} may preferentially proliferate during the process of metastasis in tumors with normal p53.

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

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