Association of p16 Homozygous Deletions with Clinicopathologic Characteristics and EGFR/KRAS/p53 Mutations in Lung Adenocarcinoma

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Abstract

Purpose: The p16 gene is frequently inactivated in lung adenocarcinoma. In particular, homozygous deletions (HD) have been frequently detected in cell lines; however, their frequency and specificity is not well-established in primary tumors. The purpose of this study was to elucidate the prevalence and the timing for the occurrence of p16 HDs in lung adenocarcinoma progression in vivo.

Experimental Design: Multiple ligation-dependent probe amplification was used for the detection of p16 HDs in 28 primary small-sized lung adenocarcinomas and 22 metastatic lung adenocarcinomas to the brain. Cancer cells were isolated from primary adenocarcinoma specimens by laser capture microdissection. HDs were confirmed by quantitative real-time genomic PCR analysis.

Results: HDs were detected in 8 of 28 (29%) primary tumors, including 2 of 8 (25%) noninvasive bronchioloalveolar carcinomas, and 5 of 22 (26%) brain metastases, respectively. No significant associations were observed between p16 HDs and gender, age, smoking history, stage, and prognosis. HDs were detected with similar frequencies (17–29%) among adenocarcinomas with epidermal growth factor receptor (EGFR) mutations, with KRAS mutations, and without EGFR/KRAS mutations, and with similar frequencies (22–28%) between adenocarcinomas with and without p53 mutations.

Conclusions: p16 HDs occur early in the development of lung adenocarcinomas and with similar frequencies among EGFR type, KRAS type, and non-EGFR/KRAS type lung adenocarcinomas. Tobacco carcinogens would not be a major factor inducing p16 HDs in lung adenocarcinoma progression.

Adenocarcinoma is the most common histologic type of lung cancer. Recent molecular analyses have indicated that lung adenocarcinoma can be divided into at least three types; the epidermal growth factor receptor (EGFR) type, the KRAS type, and the non-EGFR/KRAS type, based on accumulated genetic alterations in adenocarcinoma cells (1, 2). The p16 tumor suppressor gene is frequently inactivated in lung adenocarcinomas, most prominently through promoter methylation and homozygous deletion (HD), and less frequently through intragenic mutation (3–6). In particular, p16 methylation is known to occur in close association with tobacco carcinogen exposure and preferentially in the KRAS type adenocarcinomas (1). Molecular analyses of small-sized adenocarcinomas revealed that p16 methylation occurs in the course of progression from noninvasive bronchioloalveolar carcinomas (BAC) to invasive ones, and its occurrence is associated with smoking history, staging, and prognosis (7). However, it is still unknown whether p16 HD also occurs preferentially in the KRAS type or not. In addition, the involvement of tobacco smoking in the occurrence of p16 HD is also unclear because both positive and negative associations between tobacco smoking and HD have been reported (5, 6). Indeed, p16 HDs have been reported to occur with a highly variable frequency in primary lung adenocarcinomas (0–40%; refs. 3–6, 8). HD can be easily masked if a large fraction of noncancerous cells are contaminated in tumor tissues, in particular, in the analysis of small tumors with a BAC component. Thus, such an inconsistency could come not only from the diversity and/or heterogeneity of lung adenocarcinomas but also from tumor tissues used for the analysis and also from the methods used for the detection of HDs. For instance, immunohistochemical analyses of p16 proteins have shown that a considerable...
fraction of adenocarcinomas are negative for p16 protein expression without clear evidence of an inactivating event by molecular analyses of the p16 gene (4, 5, 7). Thus, it has been assumed that p16 HD is a causative event for the absence of p16 protein in these adenocarcinoma cases. However, due to the lack of comprehensive analysis for p16 HDs, the prevalence, specificity, and the timing for the occurrence of p16 HDs in lung adenocarcinoma progression in vivo is still unclear.

In this study, we investigated the association of p16 HDs with clinicopathologic characteristics, including smoking history, of lung adenocarcinomas and with the status of EGFR, KRAS, and p53 mutations in lung adenocarcinomas. To obtain more critical information than previous studies for the timing of its occurrence in the progression of lung adenocarcinomas, we analyzed two typical stages of lung adenocarcinomas. One is a primary small-sized adenocarcinoma of \( \leq 2 \) cm in maximum diameter as a representative of early stage lung adenocarcinomas, and the other is a brain metastasis as a representative of late stage lung adenocarcinomas. To exclude the possible overlooking of HDs in the analysis due to contamination of noncancerous cells in tumor tissues, a laser capture
microdissection method was applied for the isolation of cancer cells from small-sized primary lung adenocarcinomas. Brain metastases are known to be relatively solid and generally contain a small amount of noncancerous cells in tumor tissues; therefore, macrodissected samples were used for the analysis. Recently, a simple and effective method, which is called multiplex ligation-dependent probe amplification (MLPA), to measure the copy number of up to 45 genomic loci in a single experiment, was developed and has been applied for the detection of large deletions in/of various genes in the human genome DNA sequence (9). For instance, this method was successfully applied for the detection of p16 hemizygous deletions in melanoma families (10) and p16 HDs in head and neck squamous cell carcinoma cell lines (11). Thus, we applied this method for the detection of p16 HDs in surgically resected lung adenocarcinomas. HDs were detected with similar frequencies of 23% to 40% in lung adenocarcinomas of any progression stage—from noninvasive carcinomas to advanced ones. The deletions were also detected with similar frequencies among EGFR type, KRAS type, and non-EGFR/KRAS type adenocarcinomas, and were not associated with tobacco smoking and poor prognosis.

Materials and Methods

Patients and tissues. Twenty-eight primary small-sized (≤2 cm in maximum diameter) adenocarcinomas, 22 brain metastases, and corresponding noncancerous tissues were obtained at surgery from lung adenocarcinoma patients treated at the National Cancer Center Hospital, Tokyo and at the Saitama Medical University. In 4 of the 22 brain metastasis cases, the corresponding primary tumors were also obtained at surgery. The surgically resected specimens were fixed routinely with 10% formalin and embedded in paraffin for histologic examination. All the sections were stained with H&E and examined by light microscopy. The tumors were pathologically diagnosed according to the tumor-node-metastasis classification of malignant tumors (12).

Cell lines. Three lung adenocarcinoma cell lines, H2126, A549, and PC3, were used as positive controls of p16 HDs in MLPA and quantitative real-time genomic PCR (QRT-G-PCR) analysis. Genomic DNA were prepared as previously described (14). The primary small-sized adenocarcinomas were further classified into three types according to the histologic classification of small-sized adenocarcinoma of the lung reported by Noguchi and colleagues (13), and there were 8 type B, 15 type C, and 5 type D adenocarcinomas. These 28 tumors and corresponding noncancerous tissues were fixed with methanol and embedded in paraffin. Cancer cells were then microdissected using the PixCell Laser Capture Microdissection system (Arcturus Engineering), and their genomic DNAs were extracted as described previously (14). Representative figures of type B and C tumors before and after microdissection have been shown in our previous article (14). All of the 22 brain metastases, corresponding primary tumors, and noncancerous tissues were macrodissected and stored at −80°C until DNA extraction. Genomic DNAs were prepared as previously described (15).

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MLPA analysis. MLPA was carried out using the P024B kit for the 9p21 CDKN2A/2B region (MRC-Holland) according to the manufacturer’s protocol. The kit contains 12 probes covering the p15/p14ARF/p16 genes. 12 probes for 9 other genes on chromosome 9p, and 15 control probes for nonchromosome 9p loci. Genes on chromosome 9p included in this screen were TEK, ELAVL2, CDKN2B (p15), CDKN2A (p14ARF/p16), MTAP, IFNA1, KIAA1354, IFNW1, IFNB1, MLLT3, and DOCK8 (FLJ00026) in the order from centromere to telomere (Fig. 1A). Experiments were done in a half volume until the ligation reaction step, and then by the supplied protocol. Briefly, 12.5 to 50 ng of genomic DNA in 2.5 to 5 μL of TE buffer were heat-denatured and hybridized to probes for 16 h at 60°C. The hybridized probes were then ligated and amplified by PCR of 35 cycles at 60°C for 30 s and 72°C for 60 s. PCR products were separated by capillary electrophoresis using the ABI3700 Automated Capillary DNA Sequencer with a 50-cm capillary array, ABI POP-6 polymer, and GeneScan-ROX 500 size standards (Applied Biosystems). Analysis was automated using ABI PRISM GeneScan Analysis software version 3.7, and Genotyper Analysis software version 3.7 (Applied Biosystems). PCR and electrophoresis, respectively, were done in duplicate, and the mean of four values was calculated and considered to be the DNA copy number ratio of each locus.

Data analysis. The relative DNA copy number of each locus was calculated as follows. First, the value for the sum of 15 autosomal control peak areas in a lung adenocarcinoma sample was adjusted to the mean value for those in two or three normal lung tissue samples in the same run. Each of the 39 probe peak areas was then divided by the sum of the peak areas of the 15 autosomal control probe peak areas. Finally, the relative DNA copy number ratio of each of the 39 chromosome loci in the lung adenocarcinoma samples against the normal lung tissue samples was calculated. Theoretically, ratios close to 1.0 indicated that two DNA copies were present (i.e., wild-type), a ratio of 0.5 indicated that one copy was absent (i.e., hemizygous deletion), and a ratio of 0.0 indicated that both copies were absent (i.e., homozygous deletion). The criteria for hemizygous deletions and HDs of the 39 loci by MLPA analysis were defined using MLPA data from three lung adenocarcinoma cell lines with p16 HDs as described in Results and Discussion. The p16 gene was then defined as hemizygously deleted or not in each sample. Because the purpose of this study is to evaluate the prevalence of p16 HD, hemizygous deletions were not evaluated from MLPA data.

QRT-G-PCR analysis. TaqMan-MGB probes and primers were designed using Primer Express software (Applied Biosystems) and were optimized according to the manufacturer’s guidelines. Target and reference locus probes were labeled with FAM and VIC, respectively. Probe sequences were as follows: no. 9, 5′-AATCCTTCAGACATTC-3′; and 2p14, 5′-CCAGGCTATCCGTC-3′. Primer sequences were as follows: no. 9-F/R, 5′-GGGTCTCCTTCATTTGGTGAAA-3′; and 2p14-F/R, 5′-AAGAAAGCTGGAGTTTGGTGAAA-3′. PCR was carried out in duplicate using 1 ng of DNA as a template. Primer and probe concentrations were optimized for each target according to the manufacturer’s instructions. The PCR program consisted of 50°C for 2 min and 95°C for 15 min followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. Standard curves for the copy numbers of the target and reference genes were generated using serially diluted (0.04–25 ng) normal lung tissue DNA. Data analysis was carried out using ABI Prism 7900HT Sequence Detection Software. DNA copy number ratios were calculated as the average copy number of the target locus divided by the average copy number of the reference locus, and then normalized against the normal lung tissue DNA to give a normalized DNA copy number ratio.

Immunohistochemistry. Immunohistochemical analysis of p16 protein was performed as described previously (7) using 4-μm sections cut from methanol-fixed and paraffin-embedded specimens of 28 primary small-sized adenocarcinomas, which were subjected to MLPA analysis. Positivity for p16 staining was scored by the same criteria as previously described (7). In brief, only nuclear staining was scored, and was considered to be positive when it was more intense than the background cytoplasmic staining. If <10% of tumor cells displayed p16 protein staining, it was judged negative; and if ≥10% of them showed strong staining, it was judged positive.

Mutation analysis of the EGFR, KRAS, and p53 genes. Thirteen of the 28 primary tumors, and 16 of the 22 brain metastases were...
Table 1. Frequencies of p16 HDs and EGFR/KRAS/p53 mutations in lung adenocarcinoma

<table>
<thead>
<tr>
<th>Sample</th>
<th>Subtype</th>
<th>Frequency (%)</th>
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<tr>
<td></td>
<td>p16</td>
<td>EGFR</td>
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<tr>
<td>Surgical specimen</td>
<td>13/50</td>
<td>34/50</td>
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<tr>
<td>(26)</td>
<td>(68)</td>
<td>(8)</td>
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<tr>
<td>(29)</td>
<td>(75)</td>
<td>(7)</td>
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<td>(25)</td>
<td>(100)</td>
<td>(0)</td>
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<td>Type C</td>
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<td>12/15</td>
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<tr>
<td>(27)</td>
<td>(80)</td>
<td>(7)</td>
</tr>
<tr>
<td>Type D</td>
<td>2/5</td>
<td>1/5</td>
</tr>
<tr>
<td>(23)</td>
<td>(59)</td>
<td>(9)</td>
</tr>
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<td>13/22</td>
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<tr>
<td>(20)</td>
<td>(55)</td>
<td></td>
</tr>
<tr>
<td>Cell line*</td>
<td>20/55</td>
<td>-</td>
</tr>
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*Defined in our previous study (17).

Results and Discussion

Detection of HDs by MLPA analysis. We previously determined regions of p16 HDs in various lung cancer cell lines by multiplex genomic PCR analysis (3, 17). Thus, we first validated the sensitivity and specificity of the MLPA method to detect p16 HDs using several lung adenocarcinoma cell lines. No PCR amplification was observed for probes that hybridized to the sequences of HD regions in all the cell lines examined. Representative results of MLPA analysis for two cell lines, H2126 and PC3, are shown in Fig. 1B. We then applied MLPA analysis for the detection of p16 HDs in surgically resected adenocarcinoma samples. Cancer cells of small-sized primary adenocarcinomas were isolated by laser capture microdissection, and brain metastases generally contain a small fraction of noncancerous cells. However, a complete absence of PCR products was not observed for these samples at any chromosomal loci by MLPA analysis. Therefore, we next defined the criteria for p16 HDs by MLPA analysis for surgically resected samples. For this purpose, DNA samples with virtual hemizygous deletions were prepared by mixing the same amounts of DNA from normal lung tissue and from three lung cancer cell lines with p16 HDs, H2126, A549, and PC3. In total, 64 probe loci were homozygously deleted in these cell lines. The mean ± 3 SD of relative DNA copy number ratios for the 64 probe loci in the HD regions among these mixed samples was 0.54 ± 0.17; thus, the range of virtual hemizygous deletions was 0.37 to 0.71. Indeed, none of the 64 probe loci in the HD regions of the mixed samples showed a DNA copy number ratio of >0.71 or <0.37 (Fig. 1B). Therefore, if the DNA copy number ratio for a locus was <0.37, the locus was judged as homozygously deleted in surgically resected lung adenocarcinoma samples.

Under this criterion, one or more loci in the p15/p14ARF/p16 gene region were judged as homozygously deleted in 8 of 28 primary tumors and in 5 of 22 brain metastases (Table 1). Representative adenocarcinoma cases judged as having HDs of the same loci from surgical specimen, primary tumor, and brain metastasis, are shown in Fig. 1A. Case B-2P showed a HD of only one locus (probe no. 13) in exon 2 of the p16 gene. On the other hand, most of the other 12 cases showed HDs of several genes, including the p14ARF and/or p16 genes (Table 2). Both the invasive region (C-10P-in) and the BAC component (C-10P-BAC) of case C-10P showed HDs of the same loci from the p15 exon 1 to IFNW1 region. In case D-4P, the p15, p14ARF, p16, and MTAP genes were homozygously deleted. A metastasis to the brain, case N2131M, showed a large HD of the region from the ELAVL2 gene to the IFNB1 gene including the p16 gene. Furthermore, the relative DNA copy number ratios of the corresponding loci were also decreased in the corresponding primary tumor, N2133P, although the ratios were underrepresented as HDs. This may be attributed to a
contamination of noncancerous cells in the primary tumor sample because this sample was macrodissected but not microdissected. There was no probe loci deleted in all the cases, but six loci (probe nos. 4, 5, 6, 8, 9, and 13) were deleted in 12 of 13 cases, including exon 2 of the \( p16 \) gene (Table 2).

**Confirmation of HDs by QRT-G-PCR analysis.** QRT-G-PCR was performed to confirm \( p16 \) HDs detected by MLPA analysis. Among 15 control probes for nonchromosome 9p loci, a probe for a chromosome 2p14 locus showed the most consistent DNA copy number ratios of nearly 1.0 in surgically resected adenocarcinoma samples. Thus, two primer sets were designed for QRT-G-PCR analysis. One was for the amplification of an MLPA probe locus between the \( p14ARF \) gene and the \( p16 \) gene (probe no. 9 in Fig. 1A, and hereinafter referred to as the \( p14ARF/p16 \) locus), and the other was for the amplification of a control probe locus on chromosome 2p14.

QRT-G-PCR was carried out for 16 brain metastases and the corresponding 4 primary tumors. Among them, five brain metastases were judged as having HDs of the \( p14ARF/p16 \) locus by MLPA analysis. DNA from normal lung tissue was used as a negative control, and DNA from two lung cancer cell lines with \( p16 \) HDs, A549 and H2126, were used as positive controls. No PCR products were detected for the \( p14ARF/p16 \) locus in the A549 and H2126 cell lines (data not shown). The DNA copy number ratios of the \( p14ARF/p16 \) locus in all five cases that had been determined to have HDs of this locus by MLPA analysis were <0.45. Thus, these five cases were also judged as having less than one copy of the \( p14ARF/p16 \) gene by QRT-G-PCR analysis. We further analyzed the association between the results of MLPA analysis and those of QRT-G-PCR analysis among all the 20 cases. The correlation coefficient was 0.95, and a highly significant correlation was observed between them (\( P = 1.87 \times 10^{-10} \); Fig. 2). This result gave the agreement for the appropriateness of the criterion for \( p16 \) HDs in MLPA analysis.

**Occurrence of \( p16 \) HDs in early stage lung adenocarcinoma.** Based on the results of MLPA analysis, together with the confirmation by QRT-G-PCR analysis, we concluded that \( p16 \) HDs were present in 8 of 28 (29%) small-sized primary tumors and in 5 of 22 (23%) brain metastases (Table 1). Among four pairs of brain metastases and the corresponding primary tumors, only one metastasis (N2131M) was judged as having a \( p16 \) HD as described above and shown in Fig. 1C, and none of the remaining three cases showed HD in either brain metastases or primary tumors. Small-sized adenocarcinomas

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**Fig. 2.** Correlation between the results of MLPA analysis and those of QRT-G-PCR analysis. Relative DNA copy numbers of the probe no. 9 locus in Fig. 1A on chromosome 9 against the control locus on chromosome 2 defined by the QRT-G-PCR (X-axis) and MLPA analyses (Y-axis). Sixteen brain metastases and four of the corresponding primary tumors were analyzed. Values 0.5 by QRT-G-PCR analysis and values 0.37 by MLPA analysis (indicated by lines) were considered as having HDs.

**Fig. 3.** Histology (H&E; original magnification, \( \times100 \) for B-2P/C-12P/C-64P and \( \times40 \) for C-10P; top) of small-sized lung adenocarcinomas and immunohistochemical staining of \( p16 \) protein (original magnification, \( \times400 \); bottom) in the corresponding tissues. B-2P, C-10P, and D-12P are representative cases of type B, C, and D tumors with \( p16 \)-negative staining, and C-64P is a representative case of type C tumors with \( p16 \)-positive staining. A BAC component (BAC) and an invasive region (INV) are shown separately for C-10P.
These results strongly indicate that most p16 HDs detected in similar to those in brain metastases and cultured cell lines. primary adenocarcinomas was not significantly lower than, but lines (17). Thus, the frequency of p16 HDs in small-sized HDs were represented in 20 of 55 (36%) lung adenocarcinoma cell primary adenocarcinomas. We previously reported that p16 frequencies of 25% to 40% in noninvasive and invasive adenocarcinomas and p16 HDs were detected in 2 of 5 (40%) C-65P). Type D tumors are invasive, poorly differentiated BAC components of two type C tumors (cases C-10P and tumors and in 4 of 15 (27%) type C tumors, in particular, in type B, C, and D tumors are invasive adenocarcinomas with poorly differentiated adenocarcinomas and p16 HDs were detected in 2 of 5 (40%) of the type D tumors. Thus, p16 HDs were present with similar frequencies of 25% to 40% in noninvasive and invasive primary adenocarcinomas. We previously reported that p16 HDs were present in 20 of 55 (36%) lung adenocarcinoma cell lines (17). Thus, the frequency of p16 HDs in small-sized primary adenocarcinomas was not significantly lower than, but similar to, those in brain metastases and cultured cell lines. These results strongly indicate that most p16 HDs detected in the cell lines occurred in vivo during adenocarcinoma progression and were retained during cultivation in vitro of adenocarcinoma cell lines. Similar frequencies of p16 HDs between noninvasive and invasive adenocarcinomas and between primary and metastatic adenocarcinomas further indicate that p16 HD occurs early in the multistage carcinogenic process of lung adenocarcinomas.

**Table 3.** Associations of p16 HDs with p16 protein expression, clinicopathologic characteristics, and EGFR/KRAS/p53 mutations in lung adenocarcinoma

<table>
<thead>
<tr>
<th>Clinicopathologic characteristic and genotype</th>
<th>Subset</th>
<th>No. of cases</th>
<th>p16 HD (%)</th>
<th>P*</th>
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<tr>
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<td>0 (0)</td>
<td>13 (100)</td>
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<td></td>
<td>-</td>
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<td>17 (68)</td>
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<tr>
<td></td>
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<td>6 (27)</td>
<td>16 (73)</td>
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<td>16</td>
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<td>11 (69)</td>
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<tr>
<td></td>
<td>II-III</td>
<td>12</td>
<td>3 (25)</td>
<td>9 (75)</td>
</tr>
<tr>
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<td>+</td>
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<td>4 (21)</td>
<td>15 (79)</td>
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<tr>
<td></td>
<td>-</td>
<td>9</td>
<td>4 (44)</td>
<td>5 (56)</td>
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<tr>
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<td>24 (71)</td>
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<td>4</td>
<td>1 (25)</td>
<td>3 (75)</td>
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<td></td>
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<td>23 (72)</td>
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<td></td>
<td>Mutation (-)</td>
<td>18</td>
<td>4 (22)</td>
<td>14 (78)</td>
</tr>
</tbody>
</table>

*Fisher’s exact test.
†Defined for 28 patients with primary lung adenocarcinoma.
‡E(+), EGFR mutation (+); E(-), EGFR mutation (-); K(+), KRAS mutation (+); K(-), KRAS mutation (-).

were further classified histologically into types B, C, and D (Fig. 3). Type B tumors are noninvasive BACs and type C tumors are invasive adenocarcinomas with noninvasive BAC components. p16 HDs were detected in 2 of 8 (25%) type B tumors and in 4 of 15 (27%) type C tumors, in particular, in BAC components of two type C tumors (cases C-10P and C-65P). Type D tumors are invasive, poorly differentiated adenocarcinomas and p16 HDs were detected in 2 of 5 (40%) of the type D tumors. Thus, p16 HDs were present with similar frequencies of 25% to 40% in noninvasive and invasive primary adenocarcinomas. We previously reported that p16 HDs were present in 20 of 55 (36%) lung adenocarcinoma cell lines (17). Thus, the frequency of p16 HDs in small-sized primary adenocarcinomas was not significantly lower than, but similar to, those in brain metastases and cultured cell lines. These results strongly indicate that most p16 HDs detected in the cell lines occurred in vivo during adenocarcinoma progression and were retained during cultivation in vitro of adenocarcinoma cell lines. Similar frequencies of p16 HDs between noninvasive and invasive adenocarcinomas and between primary and metastatic adenocarcinomas further indicate that p16 HD occurs early in the multistage carcinogenic process of lung adenocarcinomas.

No p16 expression in tumors with p16 HDs. Because one of the purposes of this study was to confirm the immunohistochemical negativity of adenocarcinoma cells with p16 HDs, we further performed an immunohistochemical analysis on 28 cases of small-sized adenocarcinomas, all of which were subjected to MLPA analysis. Fifteen of the 28 cases (54%) showed negative immunoreactivity for p16 protein. As predicted, all eight cases with p16 HDs following MLPA analysis were negative for p16 protein expression (Table 3). Representative results of immunohistochemical staining for type B, C, and D tumors are shown in Fig. 3. It was noted that most tumor cells were clearly negative for nuclear p16 staining in these eight cases, and that both BAC components and invasive regions were negative in all four type C tumors with p16 HDs. This result strongly supports the reliability of MLPA analysis for the detection of p16 HDs in primary lung adenocarcinomas. Thus, it was concluded that p16 protein is not expressed in a considerable fraction of small-sized lung adenocarcinomas due to HDs of the p16 gene. The consistency of the results of immunohistochemistry with that of MLPA analysis further supports the hypothesis that p16 HD occurs early in lung adenocarcinoma progression.

Previously, Dr. Noguchi, who is one of the authors of this article, and his colleagues reported that 29 of 57 (51%) small-sized lung adenocarcinomas of types A to F were negative for p16 immunostaining (7). In their report, the frequency of p16 negativity was higher in smokers and in patients with non-BACs. Aberrant methylation of the p16 gene promoter was detected more frequently in advanced BACs (type C) and non-BAC (types D–F) than in BACs (types A and B). In this study, the overall frequency (54%) of p16 negativity was quite similar to their reports, and the frequency was also higher in smokers than in nonsmokers (9 of 13 versus 6 of 15), and in type D (5 of 5) than in type B (7 of 15) or type C (3 of 8). Thus, it was highly suggested that a majority of cases with negative p16 expression without p16 HDs could be due to methylation of the p16 gene promoter and the methylation was associated with tobacco smoking. However, methylation and HD are not likely to coexist with each other because the regions of HDs in the p16 gene promoter and the methylation was associated with tobacco smoking. However, methylation and HD are not likely to coexist with each other because the regions of HDs in the p16 gene promoter and the methylation was associated with tobacco smoking. However, methylation and HD are not likely to coexist with each other because the regions of HDs in the p16 gene promoter and the methylation was associated with tobacco smoking.
patients with lung adenocarcinomas (Table 3). No significant associations were observed between p16 HDs and gender, age, and smoking history in all 50 cases analyzed (Table 3), as well as in 28 cases of primary adenocarcinomas or in 22 cases of brain metastases (data not shown). p16 HDs were not associated with pathologic stage nor with 5-year survival in 28 cases of primary adenocarcinomas (Table 3). Previously, p16 methylation was shown to be associated with smoking history (1, 7). Thus, no association of p16 HDs with smoking history was in contrast with the status of p16 methylation in lung adenocarcinomas. The results indicate that causative factors for p16 HD were different from those for p16 methylation, although both alterations result in the inactivation of the same gene. Previously, Kraunz and colleagues reported that p16 HD occurred at a higher frequency in never-smokers as compared with former and current smokers (6). Although such an association was not observed in this study, both studies indicated the absence of a positive association between p16 HD and smoking history, and the possible association of p16 HD with other causative factors for lung adenocarcinoma. Thus, further studies are needed for the elucidation of such factors because little is known about the environmental as well as genetic risk factors for lung adenocarcinoma. The absence of any association between p16 HDs and 5-year survival was also in contrast with the status of p16 methylation in lung adenocarcinomas. However, because the number of cases examined was small (28 cases), and the patients with poor prognosis had a higher frequency of p16 HDs than those with good prognosis (44% versus 21%), further studies will be required on this subject. The absence of any association between p16 HDs and pathologic stage further supports their occurrence in the early stages, rather than in the late stages, of lung adenocarcinoma progression.

**Association of p16 HDs with EGFR, KRAS, and p53 mutations.** We next evaluated the association of p16 HDs with the status of EGFR, KRAS, and p53 mutations in these samples (Tables 1 and 3). Then, based on the results of these molecular analyses and a previous p16 methylation analysis (7), a stepwise malignant progression model for small-sized lung adenocarcinoma was depicted as shown in Fig. 4. As previously reported (14, 18), EGFR and KRAS mutations were detected in a mutually exclusive manner in lung adenocarcinomas. EGFR mutations were most frequently detected in type B tumors (8 of 8, 100%), suggesting the involvement of EGFR mutations in the formation of noninvasive BACs. Although KRAS mutations were detected only in type C and D tumors in this study, it was recently reported that the mutations were frequently detected in noninvasive adenocarcinomas and also in atypical adenomatous hyperplasias (AAH; ref. 19). Thus, it is likely that either EGFR or KRAS mutations occur prior to HDs and methylations of the p16 gene in the progression of BACs, although it is also possible that p16 alterations occur earlier than depicted in Fig. 4. Frequencies of p53 mutations in primary adenocarcinomas were the highest in type D (5 of 5, 100%), intermediate in type C (9 of 15, 60%), and the lowest in type B (2 of 8, 25%), suggesting the accumulation of the mutations during progression from noninvasive adenocarcinomas to invasive ones. Thus, it was likely that p53 mutations had accumulated in adenocarcinomas with p16 HDs and/or EGFR/KRAS mutations. It is indispensable to analyze a considerable number of AAHs as well as type A tumors to fully understand the timing of each genetic alteration in sequential progression of lung adenocarcinomas because AAH is a putative precursor of peripheral lung adenocarcinoma including BAC (20), and sequential progression from type A to type C tumors through type B tumors was strongly indicated in previous

![Fig. 4. A stepwise malignant progression model of small-sized lung adenocarcinoma in association with accumulated genetic alterations in cells. BAC, bronchioloalveolar carcinoma; type C, localized bronchioloalveolar carcinoma with foci of active fibroblastic proliferation; type D, poorly differentiated adenocarcinoma.](https://www.aacrjournals.org/clinican2008/article-pdf/14/12/3752/4554820/clinican2008_14_12_3752.pdf)
studies (7, 21). However, because AAH is not routinely resected by surgery and because type A tumors are usually very small, the number of tumors as well as the amount of DNA obtained was not enough for the present study.

There was no specificity for the occurrence of p16 HDs among adenocarcinomas with regard to other accompanied genetic alterations. In particular, p16 HDs were detected with similar frequencies (17–29%), irrespective of the presence or absence of EGFR, KRAS, and p53 mutations (Table 3). This result indicates that p16 HDs occur with similar frequencies in EGFR type as well as KRAS type and non-EGFR/KRAS type adenocarcinomas. In this model, type C tumors were considered to progress from BACs, as previously indicated by Aoyagi and colleagues (21). However, it was still unclear whether type D tumors arise de novo by a distinct pathway from tumors with BAC components or progress from these tumors. Low frequencies of EGFR mutations and high frequencies of p53 mutations in type D tumors indicate that type D tumors progress from either the KRAS types or non-EGFR/KRAS types. Haneda and colleagues also reported a low frequency of EGFR mutations in type D tumors (22), supporting the presence of a non-EGFR pathway for the development of type D poorly differentiated adenocarcinomas.

Conclusions. MLPA analysis of microdissected small-sized primary adenocarcinoma cells revealed that p16 HDs are present in 20% to 40% of adenocarcinomas irrespective of the presence of mutations in the EGFR, KRAS, and p53 genes, and occur early in the development of lung adenocarcinomas. HDs were not associated with smoking history of the patients. It has been indicated that smoking is a major factor inducing p16 methylation as well as KRAS mutation. In contrast, EGFR mutations frequently occur in female nonsmokers and are associated with bronchioloalveolar morphology of adenocarcinomas. Interestingly, p16 HDs did not coexist with specific genetic alterations in adenocarcinoma cells. Thus, causative factors for p16 HD would be different from those for p16 methylation, KRAS mutations, and EGFR mutations. To elucidate the causative role for the occurrence of p16 HDs in multistage lung carcinogenesis, further studies should focus on the identification of environmental and genetic factors for the induction of DNA double-strand breaks surrounding the p16 gene locus and their repair systems.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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