CpG Island Methylation Is Responsible for p14ARF Inactivation and Inversely Correlates with p53 Overexpression in Resected Non–Small Cell Lung Cancer

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ABSTRACT

Purpose and Experimental Design: The molecular mechanisms by which the p14ARF gene is altered in non–small cell lung cancer (NSCLC) are complex and unclear. Using genetic and epigenetic analyses, we examined various molecular alterations including the loss of protein and mRNA expression, and 5′CpG hypermethylation, allelic imbalance, and mutation of the p14ARF gene in a series of 102 NSCLC samples, in parallel with clinicopathological and prognostic analyses. To clarify the biological significance of p14ARF alterations, its relationship with p16INK4a and p53 alterations was also examined.

Results: We found that 34% of NSCLC patients had aberrant p14ARF protein expression, which was more frequent in adenocarcinomas (AD; 44%) than in squamous cell carcinomas (22%; \( P = 0.024 \)). A high concordance was observed between alterations in protein and mRNA expression and 5′CpG hypermethylation (\( P \leq 0.001 \)). The p14ARF hypermethylation inversely correlated with p53 overexpression (\( P = 0.001 \)). This mutually exclusive relationship for alteration between p14ARF and p53 was also supported by a worse prognosis of AD patients with positive P14ARF expression (\( P = 0.01 \)) and of AD patients with P53 overexpression (\( P = 0.006 \)). Our data also indicated that hemizygous/homozygous deletion and mutation in the p14ARF gene occurred at 26%, 9%, and 0%, respectively, of microdissected NSCLCs.

Conclusions: Our data suggest that p14ARF 5′CpG hypermethylation is the predominant mechanism involved in the aberrant expression of the p14ARF gene. In addition, p14ARF 5′CpG hypermethylation occurs inversely to P53 overexpression.

INTRODUCTION

The INK4a/ARF locus on human chromosome region 9p21 encodes two distinct proteins, which are translated in different reading frames from alternatively spliced transcripts (1). P16INK4a is specified by the \( \alpha \) transcript composed of exons 1a, 2, and 3. The other product, P14ARF, is encoded by the smaller \( \beta \) transcript, which is composed of exons 1\( \beta \), 2, and 3\( \beta \). It is known that P14ARF and P16INK4a are upstream regulators in the P53 and RB tumor suppressor pathways, respectively. P16INK4a inhibits cyclin-dependent kinases 4 and 6 to phosphorylate RB protein, resulting in the induction of G1 arrest (3). P14ARF prevents the MDM2-mediated degradation of P53 protein (4, 5). Thus, P14ARF overexpression leads to the stabilized P53, which then induces several biological responses against DNA damage, such as \( G_1 \) arrest, \( G_2-M \) arrest, and apoptosis (5–7). It has also been reported that the P14ARF-mediated \( G_1 \) and \( G_2 \) arrests are abolished in mouse embryonic fibroblasts lacking functional P53, indicating that P53 acts downstream of P14ARF in a cell cycle regulatory pathway (8).

Inactivation of the p16INK4a gene results from intragenic mutation, homozygous/hemizygous deletions, and promoter hypermethylation, and these genetic and epigenetic alterations have been detected frequently in a variety of human cancers (9) including non–small cell lung cancer (NSCLC; Refs. 10–12). However, the pathogenic and biological significance of p14ARF gene alterations in human cancers is still unclear. Although the p14ARF null mice (where only the exon 1\( \beta \) is lost) develop spontaneous tumors at an early age (8), no germ-line mutations affecting the p14ARF-specific exon 1\( \beta \) have been identified (13, 14), and yet many INK4a/arF exon 2 mutations alter both the P16INK4a and P14ARF amino acid sequences (15). Alteration of the P14ARF protein expression is found by immunohistochemistry in 25–41% of NSCLCs (16, 17). However, contrary results have been reported when loss of P14ARF expression was correlated with abnormal \( \beta \) transcripts (16, 17). Various loss of heterozygosity (LOH) assays using different microsatellite markers have indicated allelic loss in the 9p21 at various frequencies (12, 18, 19). In two studies of NSCLC, homozygous deletion of p14ARF was indirectly inferred from allelotype pattern at 9p21 (18) or from amplification of a fragment containing noncoding intronic sequence (20), but this was not confirmed in other studies. Sequence analysis revealed that exon 1\( \beta \) mutation occurs rarely in the p14ARF gene (16, 18, 20).
In data published to date, only a few reports showed p14ARF methylation in 5–9% of primary NSCLCs (21–23). However, the correlation of p14ARF methylation with protein expression was not examined in these studies.

The frequency and mechanisms of p14ARF gene inactivation and their correlation with loss of protein expression in NSCLCs vary between different studies. Furthermore, data concerning the frequency and mechanisms of p14ARF gene inactivation have rarely been documented in the same series of NSCLC. To elucidate the possible mechanisms involved in p14ARF changes in NSCLC tumorigenesis, we performed a comprehensive genetic and epigenetic study of the p14ARF status in a series of 102 NSCLC samples and compared the data with the clinicopathological parameters and prognosis of the patients. To clarify the biological significance of p14ARF alterations, we also analyzed the relationship of p14ARF alterations with p53 and p16INK4a alterations. The results indicated that p14ARF alteration is involved in NSCLC tumorigenesis and mainly results from 5’CpG hypermethylation. In addition, p14ARF hypermethylation inversely correlated with P53 over-expression and occurred frequently in tumors with p16INK4a hypermethylation.

MATERIALS AND METHODS

Sample Preparation and Clinical Characterization of Patients. Tissues were collected after obtaining permission from the appropriate Institutional Review Board and informed consents from the recruited patients. Surgically resected tumor samples from 102 patients with NSCLC were collected between 1999 and 2001. Of these patients, 54 had squamous carcinoma (SQ), 42 had adenocarcinoma (AD), 2 had adenosquamous cell carcinoma (AS), and 4 had large-cell carcinoma (LC). The histology of the tumor types and their stages were determined according to the WHO classification and the Tumor-Node-Metastasis system, respectively. Information on the smoking history of the patients with lung cancer was obtained from hospital records. The patients were classified into smoking and ex-smokers. Follow-up of the 92 patients was performed at various hospitals and clinics in Japan. The end of the follow-up period was defined as June 2003. For the 51 patients who died during the follow-up period, the mean follow-up time was 30 months (range, 24–51 months). For the 41 patients who survived the follow-up period (censored patients), the mean follow-up time was 30 months (range, 24–51 months). For the 51 patients who died during the follow-up period, the mean follow-up time was 13 months (range, 0.5–31 months).

Surgically resected tumor samples were immediately snap-frozen and subsequently stored in liquid nitrogen. For the methylation assay, genomic DNA was extracted according to the standard methods described above. For the RNA expression assay, the total RNA were prepared from matched pairs of primary tumors and nearby normal lung tissues using Trizol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized using SuperScript reverse transcriptase (Invitrogen) with the protocols provided by the manufacturer.

Analysis of Protein Expression: Immunohistochemistry Assay. Paraffin blocks of tumors were cut into 5-μm slices and then processed using standard deparaffinization and rehydration techniques. Polyclonal antibody p14ARF/p16β Ab-4 (1:100; NeoMarkers, Union City, CA) was used as the primary antibodies to detect P14ARF protein expression. The primary antibody was detected using biotinylated secondary antibody (DAKO, Carpinteria, CA) according to the manufacturer’s instructions. The sections were then counterstained with hematoxylin. The stains were graded negative when there was complete absence of staining in the tumor cell nuclei, with adequate nuclear staining in surrounding normal stromal and epithelial cells. The samples were assayed in batches including both negative- and positive-expression patients. The analysis for the P53 expression was described previously (24).

Analysis of mRNA Expression: Multiplex Reverse Transcription-PCR (RT-PCR) Assay. The RNA was extracted, and 96 samples had adequate RNA for additional analysis. p14ARF mRNA expression was assayed in a multiplex RT-PCR analysis using the β-actin gene as an internal control. The coding regions of exons 1–2 of the P14ARF gene and the β-actin gene were amplified using primers described by Gazzeri et al. (16). Reactions were carried out in a volume of 25 μl with 1 μl cDNA and 0.25 pmol primers on a DNA thermal cycler. PCR was performed for 35 cycles with an annealing temperature of 70°C. The number of cycles and the amount of primers and the cDNA used were determined to provide quantitative amplification during multiplex RT-PCR. To quantify the relative levels of gene expression in the multiplex RT-PCR assay, the value for the internal standard (β-actin) in each test tube was used as the baseline value for gene expression in that sample, and a relative value was calculated for each target p14ARF transcript amplified from each tumor and matched normal sample. Tumor cells that exhibited mRNA expression below 50% of that of normal cells were deemed to have an abnormal pattern.

Analysis of 5’CpG Hypermethylation: Restriction Enzyme-Based Multiplex PCR (RE-PCR) Methylation Assay and Methylation-Specific PCR (MSP) Assay. Samples for the methylation assay were selected on the basis that they were composed of >70% tumor tissue when observed within each of the resected tissue sections at low-power (×100) magnification under a light microscope. Therefore, the methylation assay was performed with 91 samples. The 5’CpG methylation status of the p14ARF and p16INK4a genes were investigated using the BstUI- and Smal-based multiplex PCR analyses, respectively. The analysis of Smal-based multiplex PCR for the p16INK4a hypermethylation was described previously (12). Genomic DNA from 91 tumor samples (250 ng) were digested either with 3 units of the methylation-sensitive enzyme (BstUI; New England Biolabs, Beverly, MA) or placed in the appropriate buffer without an enzyme for 16 h at 37°C. The DNA was subjected to a second round of digestion by another 3 units of
freshly added enzymes to improve specificity and completeness of digestion. The digested DNA was purified by phenol-chloroform extraction followed by ethanol precipitation. Fifty ng of the purified DNA were subjected to the multiplex PCR. The primer nucleotide sequences and thermocycling temperatures were as follows: for the 5′ CpG at the exon 1 of p14ARF gene, sense-5′GCTCACCTCTGTGCAAAAGGC3′; antisense-5′CTGCCCTAGCCGTGCTTCT3′; for the internal control IFNβ1 sequence, which is located distal to the p14ARF gene and contained no BstUI site, sense-5′ATGAGCTACAACITGCTTGGAA3′, antisense-5′TCGGTTTCGAGGTAAACCTTG3′. PCR was performed for 40 cycles with an annealing temperature of 66°C. In addition, DNA from normal lymphocytes and Xes methylase-treated DNA were included in each assay to serve as unmethylated and methylated controls, respectively. An aliquot of each PCR product was resolved on a 1.5% agarose gel.

The methylation status of the p14ARF gene of 37 tumor samples was also determined by chemical treatment with sodium bisulfite and subsequent methylation-specific PCR (MSP) analysis as described (22). PCR was performed for 40 cycles with the annealing temperature of 65°C using 60 ng bisulfite-modified DNA and 1 unit of AmpliTaq Gold polymerase (PE Applied Biosystems, Foster City, CA). All of the PCRs were performed with positive controls for both unmethylated and modified DNA and 1 unit of AmpliTaq Gold polymerase (PE Applied Biosystems). Sequencing was performed on both DNA strands.

**RESULTS**

**p14ARF Protein Expression in Primary NSCLCs and Its Correlation with Clinicopathological Parameters in NSCLC Patients.** Immunohistochemical staining was performed on 102 tumor samples. Staining within the nucleus of tumor cells was considered positive. Sixty-seven lung tumors showed staining of moderate to strong intensity (Fig. 1, A and C). The remaining 35 lung cancers (34%) showed a complete absence of nuclear staining of P14ARF protein (Fig. 1, B and D). χ² analysis indicated a highly significant correlation between aberrant P14ARF expression and histological type, recorded as negative staining in 44% (19 of 42) of NSCLC with AD compared with 22% (12 of 54) of NSCLC with SQ (P = 0.04; Table 1).

**p14ARF mRNA Expression and Its Correlation with Clinicopathological Parameters of NSCLC Patients.** RNA with high quality for multiplex RT-PCR analysis was extracted from 96 tumors (Fig. 2). Decreased p14ARF transcripts were shown by RT-PCR to occur in 30 tumors (31%; Table 1). Low mRNA expression appeared to be more frequent in AD (38%) than in SQ (25%), although it did not reach the statistical significance (P = 0.161; Table 1).

**p14ARF 5′ CpG Hypermethylation in Primary NSCLCs and Its Correlation with p16INK4a Hypermethylation and P53 Overexpression.** We studied 5′ CpG hypermethylation of the p14ARF gene in 91 tumors using the RE-PCR methylation assay (Fig. 3A). As indicated in Table 1, 30% (27 of 91) of lung tumors showed hypermethylation of the 5′ CpG region of the p14ARF gene. In addition, we performed an MSP assay for the p14ARF gene in tumors from 37 patients (Fig. 3B). The data show a 78% concordance of the methylation status for the p14ARF gene (P = 0.001), using both RE-PCR and MSP assays.

Because p16INK4a 5′ CpG hypermethylation was also
Table 1  Alterations of p14ARF gene in relation to clinicopathological parameters of resected primary NSCLCs

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Protein expression</th>
<th>mRNA expression</th>
<th>5’CpG methylation</th>
<th>LOH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>+</td>
<td>−</td>
<td>Total</td>
</tr>
<tr>
<td>Overall†</td>
<td>102</td>
<td>67</td>
<td>35</td>
<td>34%</td>
</tr>
<tr>
<td>Age (mean ± SD)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>85</td>
<td>55</td>
<td>30</td>
<td>35%</td>
</tr>
<tr>
<td>Female</td>
<td>17</td>
<td>12</td>
<td>5</td>
<td>29%</td>
</tr>
<tr>
<td>Smoker</td>
<td>76</td>
<td>51</td>
<td>25</td>
<td>33%</td>
</tr>
<tr>
<td>Nonsmoker</td>
<td>26</td>
<td>16</td>
<td>10</td>
<td>39%</td>
</tr>
<tr>
<td>Tumor type</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SQ</td>
<td>54</td>
<td>42</td>
<td>12</td>
<td>22%</td>
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<tr>
<td>AD</td>
<td>42</td>
<td>23</td>
<td>19</td>
<td>44%</td>
</tr>
<tr>
<td>AS</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>100%</td>
</tr>
<tr>
<td>LC</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>50%</td>
</tr>
<tr>
<td>Tumor stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I+II</td>
<td>51</td>
<td>32</td>
<td>19</td>
<td>37%</td>
</tr>
<tr>
<td>III+IV</td>
<td>51</td>
<td>35</td>
<td>16</td>
<td>31%</td>
</tr>
</tbody>
</table>

† NSCLC, non-small cell lung cancer; LOH, loss of heterozygosity; SQ, squamous carcinoma; AD, adenocarcinoma; AS, adenosquamous cell carcinoma; LC, large-cell carcinoma.

P14ARF protein (−) was defined as patients with an absence of nuclear staining of the P14ARF protein.

p14ARF mRNA (−) was defined as <50% of the expression level in tumor sample compared to that in its normal counterpart.

p14ARF 5’CpG hypermethylation (+) was defined as >19% of tumor DNA with DNA methylation. The cutoff value was determined by the average of normal DNA.

p14ARF LOH (+) was defined as a reduction of ≥50% in the DNA of one of the tumor sample alleles when compared with a heterozygous normal tissue control. Only informative cases (heterozygous status in normal) were included, though 91 samples were analyzed.

Total number of samples in certain categories are less than the overall number analyzed, because the clinical data and/or molecular analysis were not available for some samples.

Of the 91 samples analyzed, 81 were informative cases that showed two distinguishable alleles of different sizes in the normal DNA.

P = 0.024 by Pearson X² test between SQ and AD.

Fig. 1 Immunohistochemical analysis of p14ARF in formalin-fixed, paraffin-embedded sections of resected tumor specimens of the non-small cell lung cancer. Nuclear immunoreactivity is considered positive and is visible as a precipitate. Positive immunoreactivity in stromal cells of resected tumor specimens serves as an internal control in A–D. Adenocarcinomas (A) and squamous carcinoma tumors (C) showed positive staining. No immunoreactivity was visible in adenocarcinomas (B) or squamous carcinoma tumors (D). Original magnification, ×200.
Alterations in the reverse transcription-PCR assay. \( p14\text{ARF} \) indicated above the gel illustration. The concordant expression between protein and mRNA was also indicated above the gel illustration. \( \beta\)-actin was used as the internal control for the analysis.

![Fig. 2 Representative figures for \( p14\text{ARF} \) mRNA expression analysis by the reverse transcription-PCR assay. \( N \), normal lung tissue; \( T \), tumor tissue of the lung. Patients 26, 47, and 56 were positive for \( p14\text{ARF} \) mRNA expression. Patients 42 and 61 were negative for \( p14\text{ARF} \) mRNA expression. The concordant expression between protein and mRNA was also indicated above the gel illustration. \( \beta\)-actin was used as the internal control for the analysis.](image)

Demonstrated in this cohort of NSCLC previously (12), the correlation of methylation status of \( p14\text{ARF} \) was examined with all of the analyses, including protein and DNA methylation. The data indicated that 58 of 81 tumors had \( p14\text{ARF} \) hypermethylation with the \( P53 \) gene; \(-\) with \( 5'\)CpG hypermethylation for the \( p14\text{ARF} \) gene. Furthermore, aberrant mRNA expression was significantly associated with hypermethylation of the \( p14\text{ARF} \) gene (\( P < 0.001 \)).

\( \text{LOH at D9S942 in Resected Tumors.} \) We investigated whether LOH occurred in this series of NSCLCs. Microdissected genomic DNA obtained from 91 (81 informative) matched pairs of primary tumors and nearby normal lung tissue was examined for the incidence of LOH at the microsatellite markers D9S942 and D9S925, which are located at the chromosome region harboring the \( p14\text{ARF} \) gene. The statistical

![Fig. 3 Representative figure of \( 5'\)CpG hypermethylation analysis of \( p14\text{ARF} \) gene using the restriction enzyme-based multiplex PCR methylation assay (A) and methylation-specific PCR assay (B). A. "++", BsrUI digest. "-", mock digest. Positions of the target \( p14\text{ARF} \) \( 5'\)CpG region and \( I\gamma N\beta \) internal control are indicated. Patients 15, 37, and 60 showed \( 5'\)CpG hypermethylation of the \( p14\text{ARF} \) gene. The concordant results of mRNA and protein were also indicated above the gel illustration. Normal lymphocytes and SstI-treated DNA contain unmethylated and methylated \( p14\text{ARF} \), respectively. B, methylation-specific PCR assay is shown for 5 patients and the controls. Primer sets used for amplification are designated as \( U \) for unmethylated or \( M \) for methylated genes. All of the primary tumors included amplification with the \( U \) primer set, a result of the presence of nonnal contaminating tissue.](image)

<table>
<thead>
<tr>
<th>Protein</th>
<th>mRNA</th>
<th>Methylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>--</td>
<td>+</td>
</tr>
<tr>
<td>37</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>60</td>
<td>--</td>
<td>++</td>
</tr>
<tr>
<td>76</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>83</td>
<td>++</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 2 Comparison of \( 5'\)CpG hypermethylation in \( p14\text{ARF} \) with alterations in \( p16\text{INK4a} \) and \( p53 \) genes of NSCLC patients

<table>
<thead>
<tr>
<th>( p14\text{ARF} ) hypermethylation</th>
<th>Class</th>
<th>(+)</th>
<th>(-)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( p16\text{INK4a} ) hypermethylation</td>
<td>+</td>
<td>16</td>
<td>43</td>
<td>0.407</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>4</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>( P53 ) overexpression</td>
<td>+</td>
<td>4</td>
<td>35</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>19</td>
<td>23</td>
<td></td>
</tr>
</tbody>
</table>

\( a \) NSCLC, non-small cell lung cancer.
\( b \) ++: with \( 5'\)CpG hypermethylation for the \( p14\text{ARF} \) and \( p16\text{INK4a} \) genes, with overexpression for the \( P53 \) protein; -: without \( 5'\)CpG hypermethylation for the \( p14\text{ARF} \) and \( p16\text{INK4a} \) genes, with negative expression for the \( P53 \) protein.

![Fig. 4 Concordance analysis among protein expression, mRNA expression, and \( 5'\)CpG methylation of the \( p14\text{ARF} \) gene. The percentage of cases is indicated on the \( X \) axis, whereas the type of comparison is plotted on the \( Y \) axis. "++" indicates positive protein expression (protein), positive mRNA expression (RNA), and DNA hypermethylation (methyl), as opposed to "-", which indicates a negative result. Numbers above the bars indicate the percentage in the total concordant group (gray section) and nonconcordant group (white section). \( P \) values for the association between categories are all < 0.001.](image)
analyses showed close correlation between the LOH of these two microsatellite markers, suggesting that LOH at these markers truly represents the frequency of allelic imbalance in the region where they are located. Twenty-one (26%) of the 81 informative cases were found to harbor LOH at these markers (Table 1). LOH tended to be more frequently associated with P14ARF-negative patients than P14ARF-positive patients ($P = 0.09$).

**Homozygous Deletion and Intragenic Mutation of the p14ARF in Resected Tumors.** Homozygous deletion of the p14ARF gene was analyzed in 55 microdissected tumors, using comparative multiplex PCR for exon 1 and exon 2 regions. Five patients (9%) showed absence of exon 1 of the p14ARF during the multiplex PCR assay (Fig. 5). In the remaining tumors without homozygous deletion of the exon 1 region of p14ARF, no mutation was found by sequencing analysis. In addition, we did not detect any tumors with homozygous deletion in exon 2 of the gene. One case showed a complex mutation at bases 537–541 (codons 179 and 180) of the exon 2 region, which was described previously (12). Therefore, the mutation frequency of the p14ARF gene was 2% (1 of 55).

**Correlation of P14ARF Alteration with Prognosis of NSCLC Patients.** The relationship between postoperative survival and the negative P14ARF expression was analyzed. P14ARF protein expression was not statistically associated with prognosis ($P = 0.59$; Fig. 6A). We then analyzed the prognostic significance of P14ARF expression by stratifying the various clinicopathological parameters of patients. The survival rates in AD patients with positive P14ARF expression were significantly lower than those patients without expression ($P = 0.01$; Fig. 6B). Median survival time was 16 months for P14ARF-positive patients, whereas it was not reached for the P14ARF-negative patients. To test the hypothesis that the worse prognosis of AD patients with positive P14ARF expression is due to its inverse correlation with P53 overexpression, the relationship between survival and overexpression of the P53 was also analyzed. The data indicated that AD patients with P53 protein overexpression were also statistically associated with a poorer prognosis ($P = 0.006$; Fig. 6C).

**DISCUSSION**

In this study, a comprehensive genetic and epigenetic analysis of the patterns of inactivation of the p14ARF gene was conducted with 102 primary NSCLCs. Aberrant P14ARF protein and mRNA expression and hypermethylation of the 5’CpG region of the p14ARF gene was identified in 34%, 31%, and 30% of patients. A high concordance was observed between alterations in protein and mRNA expression and 5’CpG hypermethylation of the p14ARF gene. The data suggest that DNA hypermethylation is the predominant mechanism involved in the deregulation of the p14ARF gene. Patients with 5’CpG hypermethylation of the p14ARF gene were also hypermethylated at

![Fig. 5](#)

**Fig. 5** Representative figures of comparative multiplex PCR for the detection of homozygous deletion of exon 1 of the p14ARF gene in microdissected DNA from three tumors. Position of the p14ARF gene and 1063.7 internal controls are indicated. Homozygous deletion was seen in exon 1 of case 61.

![Fig. 6](#)

**Fig. 6** The Kaplan-Meier survival curves with respect to the P14ARF (A and B) and the P53 (C) protein expression of non-small cell lung cancer patients. $P$ values were calculated using the log rank test. A, P14ARF in all patients analyzed. The estimated median survival times for patients with negative (−) and positive (+) were 33 and 20 months, respectively ($P = 0.59$). B, P14ARF in patients with adenocarcinomas (AD). The estimated median survival time for patients with positive (+) was 16 months, whereas it was not reached for patients with negative (−). The survival rate in AD patients with P14ARF positive was markedly lower than in those with P14ARF negative ($P = 0.01$). C, the p53 overexpression group had poorer prognoses than the p53 normal expression group ($P = 0.006$). Median survival times were 16 months for p53 overexpression patients (+), whereas this was not reached for p53 normal patients (−).
the 5′CpG region of the p16INK4a gene, suggesting that 5′CpG hypermethylation plays a major role in the alteration of the INK4a/ARF locus. Our data also indicates an inverse association of p14ARF and p53 alterations in NSCLC, strongly suggesting that P14ARF and P53 act onto the same pathway.

5′CpG hypermethylation that leads to gene silencing of the p14ARF has been described in bladder, colorectal, hepatocellular, and gastric cancers in the range of 17−35% (22, 25−27), but reports are quite limited regarding primary lung cancers. The frequency of 5′CpG hypermethylation of the p14ARF gene reported in this study was 30% (27 of 91) by the RE-PCR assay. However, Zöchbauer-Müller et al. (21) and Esteller et al. (22) found frequencies of 8% (9 of 107) and 5% (1 of 20), respectively, for the p14ARF 5′CpG hypermethylation in NSCLC using the MSP assay. There seems to be at least three possible explanations for the discordance between the last mentioned two studies and the present study. First, the lower methylation frequency detected by the MSP assay compared with the RE-PCR assay may partly result from the fact that the regions examined in the two assays were different and the methylation frequency of the various CpG sites may have been different. In the present study, both RE-PCR and MSP assays were used to examine the methylation status of the p14ARF gene. A high concordance of the methylation status for the hMLH1 gene between the two assays was observed. Therefore, we considered the difference of regions examined to be an unlikely explanation for the discordance between the two assays. Secondly, the ethnic difference may also account for the difference between the three studies. Finally, the varying results may be due, in part, to differences in the types and/or stage of tumors analyzed in the contrasting studies.

5′CpG hypermethylation of p16INK4a, which is another gene located in the INK4a/ARF locus, has also been reported in many cancers by others and our group (9–12). The question regarding whether adjacent p16INK4a 5′CpG hypermethylation would be related to 5′CpG hypermethylation of the p14ARF gene was also examined in this study. Our data indicated that 80% (16 of 20) of the patients showing p14ARF 5′CpG hypermethylation were also exhibiting 5′CpG hypermethylation at the p16INK4a gene, suggesting that 5′CpG hypermethylation plays a major role in alteration of the INK4a/ARF locus. It is possible that these tumors belong to the so-called CpG island methylator phenotype group and have many genes of which the expression is down-regulated because of aberrant 5′CpG methylation. More candidate gene approaches and genomic screening techniques that permit simultaneous analysis of 5′CpG methylation of many genes should lead to a more complete knowledge of the epigenetic events occurring in tumors and its functional consequences.

In many cancers, biallelic inactivation of tumor suppressor genes is observed (28, 29). This two-step process is reflected by the LOH of polymorphic markers linked to the suppressor gene locus. We looked for such LOH at two microsatellite markers located at the chromosomal region that harbors the p14ARF gene, and examined its correlation with the status of P14ARF expression and 5′CpG hypermethylation. Eighteen samples with no expression of P14ARF protein clearly showed the retention of both alleles and DNA hypermethylation, indicating that biallelic inactivation of p14ARF expression was induced by 5′CpG hypermethylation. Nine samples lacking P14ARF expression but exhibiting aberrant methylation and LOH in the p14ARF gene suggest that biallelic inactivation of the p14ARF gene resulted from the loss of one allele and 5′CpG methylation in the remaining allele. Six tumors with no demonstrable mRNA expression and no evidence of 5′CpG methylation and LOH, other mechanisms such as mutations in promoter region of the gene, or dysfunction of the regulatory proteins could conceivably be involved in the negative mRNA expression. In addition, 2 patients with positive protein and mRNA expression showed promoter methylation. This could be explained by the presence of several distinct tumor subpopulations, one of which has methylation and does not express the gene, the others having no methylation and expressing the gene. After re-evaluating the slides from the immunohistochemistry analysis, these patients showed only 1−10% of their cells having positive stained nuclei, which was considered low P14ARF expression. Alternatively, partial methylation at only some potential sites or methylation of only one allele in these tumors may result in the positive mRNA/protein expression observed. Our results also indicated that 5 patients with homozygous deletion exhibited no P14ARF expression. The intragenic mutation was not a major cause of p14ARF inactivation in primary NSCLC, which is in agreement with the data reported by others (16, 18, 20). The single mutation in 1 patient resulted in one base substitution and four-base deletion. This complex mutation also changes the fourth ankyrin repeat consensus sequence of P16INK4a protein (12).

We observed more frequent changes in p14ARF expression in lung AD than in lung SQ. This may be partly explained by its inverse correlation with p53 alteration, which has been shown to be associated with SQ type of NSCLC in our previous study (24) and many other studies (30, 31). In addition, p14ARF alteration can be potentially used as a prognostic factor in lung AD. The poor prognosis of AD patients with positive P14ARF expression may result from their association with P53 overexpression, which has been shown to be a poor prognosis factor in AD type of NSCLCs in the present study and other studies (31, 32). We also observed an inverse correlation of p14ARF hypermethylation with p53 alteration. The data are consistent with the reports by Vonlanthen et al. (17) and by Xue et al. (33), which showed a preferential occurrence of loss of P14ARF expression in NSCLC without P53 overexpression. However, several studies observed an absence of the inverse correlation between P14ARF expression and P53 overexpression (18, 22). Two proposed explanations for the conflicting results are that selection pressures for alteration in the P53-P14ARF pathway may vary in different tumors, and cancers of different stages were analyzed in various studies. It is possible that functional dysregulation of both p53 and p14ARF genes occurred in a subset of NSCLC.

In conclusion, negative protein expression, aberrant mRNA expression, hypermethylation of the 5′CpG region, and LOH of the p14ARF gene were identified in 34%, 31%, 30%, and 26% of NSCLC patients, respectively, indicating that p14ARF deregulation is involved in NSCLC carcinogenesis. Our data add support to the premise that P14ARF is a tumor suppressor in NSCLC carcinogenesis and show for the first time a frequent p14ARF 5′CpG hypermethylation in lung cancer and an inverse
correlation of p14ARF hypermethylation and P53 overexpression in NSCLC tumorigenesis.

REFERENCES


2049

CpG Island Methylation Is Responsible for *p14ARF* Inactivation and Inversely Correlates with p53 Overexpression in Resected Non–Small Cell Lung Cancer

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