Methylation of AKAP12α Promoter in Lung Cancer

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Abstract. AKAP12α plays an important role in tumour growth suppression by inducing apoptosis. This study investigated whether the promoter methylation of AKAP12α is associated with lung cancer. AKAP12α was down-regulated in lung cancer cells and the reduced protein expression was restored by DNA methyl-transferase inhibitor. AKAP12α promoter was more frequently methylated in tumours than in normal tissues. Furthermore, AKAP12α methylation was found more frequently in the cells of non-relapse patients after surgery than in those of early relapse patients. In conclusion, this study demonstrated that AKAP12α expression is regulated by DNA methylation and that AKAP12α promoter methylation is associated with lung cancer prognosis.

AKAP12α is one of the three A-kinase anchoring protein 12 (AKAP12) isoforms that are differentially regulated by distinct promoters (1). AKAPs are members of a diverse family of scaffold proteins that possess a characteristic binding domain for the regulatory subunit of protein kinase A and have critical roles in the cell signalling pathway (2). AKAP12 isoforms, including AKAP12α, play an important role in tumour growth suppression by inducing apoptosis with the regulation of multiple molecules in the cell cycle progression (3, 4). This function of AKAP12 gene might be inhibited by epigenetic repression such as other tumour suppressor genes (4-6). DNA methylation is one of the most commonly occurring epigenetic events taking place in human carcinomas. DNA methylation typically occurs at CpG sites which are designated as CpG islands (CGI). CGIs are actually located at various positions throughout certain genes (7). The methylation of these CGIs is well known in the promoter of tumour suppressor genes in carcinogenesis, inducing gene silencing (6, 7). Recently, there has been evidence for the existence of the extracellular DNA and circulating cell-free DNA in the blood of cancer patients. These DNAs are now believed to originate from cancer cells because they contain a number of cancer-specific entities, including oncogenes, tumour suppressor genes and aberrant methylation (8-10).

In a previous report (13), AKAP12α inactivation was shown to be associated with the promoter methylation in lung cancer. However, that study was performed for a small number of lung cancer tissues and only RNA levels of the AKAP12α gene were analysed using reverse-transcription polymerase chain reaction (RT-PCR). In the present study, therefore, both in vitro methylation assay and protein assay were performed to investigate whether promoter methylation of the AKAP12α gene affects the expression of AKAP12α protein. Methylation profiling of AKAP12α promoter was also performed using the bisulfite-sequencing in lung cancer tissues, matched normal tissues and serum samples.

Materials and Methods

Cells and tissues. Four non-small cell lung carcinoma (NSCLC) cell lines (H358, H460, Calu-1 and H1299) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were grown in RPMI-1640 (Gibco/BRL, Grand Island, NY, USA) supplemented with 10% foetal bovine serum, and incubated in 5% CO2 at 37°C according to the manufacturer’s instructions.

Twenty-two samples of paired lung tumour and matched healthy lung tissues were obtained from the Korea Lung Tissue Bank of Korea University. Lung tissue-matched serum samples were acquired from the Genomic Research Center for Lung and Breast/Ovarian Cancers.
of Korea University. This study was approved by the Institutional Review Board of the Korea University Anam Hospital. Informed consent was obtained from all study participants.

**Drug treatment and Western blot analysis.** Cell lines were grown for 48 and 72 h in the presence or absence of 10 μM 5-aza-2-deoxycytidine (5-Aza-2 dc), a known DNA methyltransferase inhibitor (Sigma, St. Louis, MO, USA), and then analysed for AKAP12 protein expression by Western blot analysis, as described below.

Cells were washed in cold phosphate-buffered saline (PBS) and harvested in lysis buffer containing protease inhibitor cocktails. Cellular protein was quantified by Bradford assay (Bio-Rad, Hercules, CA, USA), and 30 μg of the cleared lysates were separated on a 6% SDS-PAGE and electro-transferred onto PVDF membranes (Amersham, Arlington Heights, IL, USA). PVDF membranes were incubated with polyclonal antibodies against AKAP12, a gift from Dr. JD Scott (Vollum Institute, Portland, OR, USA). Detection was performed using peroxysdase-conjugated secondary antibodies (Amersham) and chemiluminescence detection kit (ECL, Amersham).

**Reporter gene constructs and in vitro methylation assay.** Promoter regions of AKAP12α promoter were amplified by PCR and fused to upstream of the firefly luciferase gene in the pGL3-Basic vector (Promega, Madison, WI, USA). A series of constructs were made with the 3’ end terminating at +24 relative to the designated transcriptional start site (GenBank accession no. AF151530). The 5’ ends began at bases –837 and –443, respectively. Reporter gene constructs were confirmed by restriction mapping and sequencing. Whole AKAP12α reporter plasmids were methylated using SssI methylase (New England Biolabs, Beverly, MA, USA). To confirm the methylation, the methylated and mock-methylated plasmids were digested with a mixture of methylation-sensitive restriction enzyme, HpaII. The unmethylated (mock-methylated) or methylated AKAP12α promoter/firefly luciferase fusion genes (1 μg DNA) were transfected to H1299 cells using Lipofectamine 2000 (Invitrogen, Indianapolis, IN, USA). In each experiment, the β-galactosidase plasmid (100 ng DNA) was co-transfected for normalisation proposes. Luminescence was measured 48 h after transfection using luminometer. Reporter activity was normalised by calculating the ratio of luminescence values. Each construct was tested in three independent transfections.

**Bisulfite-sequencing analysis.** The methylation status of the AKAP12α promoter was examined using sodium bisulfite genomic sequencing, as previously reported (13). Briefly, genomic DNA was isolated using QIAamp DNA Blood Mini Kit (QIAGEN Inc., Valencia, CA, USA) and PUREGENE Kit (Genbank accession no. AF151530). The 5’ ends began at bases –837 and –443, respectively. Reporter gene constructs were confirmed by restriction mapping and sequencing. Whole AKAP12α reporter plasmids were methylated using SssI methylase (New England Biolabs, Beverly, MA, USA). To confirm the methylation, the methylated and mock-methylated plasmids were digested with a mixture of methylation-sensitive restriction enzyme, HpaII. The unmethylated (mock-methylated) or methylated AKAP12α promoter/firefly luciferase fusion genes (1 μg DNA) were transfected to H1299 cells using Lipofectamine 2000 (Invitrogen, Indianapolis, IN, USA). In each experiment, the β-galactosidase plasmid (100 ng DNA) was co-transfected for normalisation proposes. Luminescence was measured 48 h after transfection using luminometer. Reporter activity was normalised by calculating the ratio of luminescence values. Each construct was tested in three independent transfections.

**Statistical analysis.** The statistical difference between groups was analysed by paired sample Student’s t-test with SPSS version 10 (SPSS Inc., Chicago, IL, USA). Differences were considered significant if the p-value was <0.05.

**Results**

**Correlation between AKAP12α expression and methylation in lung cancer cells.** In a previous study (13), the mRNA level of the AKAP12α gene was shown to be down-regulated in lung cancer cells. To confirm the AKAP12α protein level in lung cancer cells, the present study performed Western blot assay in H358, H460, Calu-1 and H1299 cells. As shown in Figure 1A, the expression of AKAP12α protein was down-regulated in H358, H460 and Calu-1 cells, and but not in H1299 cells, consistent with the previous results of RT-PCR (13). The reduced expression of AKAP12α protein, however, was significantly alleviated by the treatment of the cells with DNA methyltransferase inhibitor, 5-Aza-2-dC (Figure 1B). These results showed that there is a correlation between methylation of CpG islands of the AKAP12α promoter and down-regulation of AKAP12α protein expression.

**Effect of methylation on the activity of AKAP12α promoter.** To further verify that promoter methylation plays a pivotal role in the regulation of AKAP12α expression, the promoter region of the AKAP12α gene was cloned and its activity was analysed under methylated or unmethylated conditions of H1299 cells using the in vitro methylation assay. Two fragments (–837–443 to the transcriptional start site) of the promoter sequence were, therefore, constructed in the
luciferase reporter vector and transfected to H1299 cells, and luciferase activity was measured as an indication of AKAP12α promoter activity. As shown in Figure 2, low luciferase activities were detected when H1299 cells were transfected with AKAP12α fragments pGL3-837 M (methylated) or pGL3-443 M (methylated), compared to the unmethylated constructs. Thus, these results indicated that promoter methylation is able to induce the gene silencing of AKAP12α expression.

**Table 1.** Comparison of CpG island methylation with clinicopathological features in lung tissues.

<table>
<thead>
<tr>
<th>Methylated CpG sites</th>
<th>Patients, no.</th>
<th>&lt;40%</th>
<th>≥40%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>18</td>
<td>56.3</td>
<td>10.1</td>
</tr>
<tr>
<td>Female</td>
<td>14</td>
<td>43.8</td>
<td>4.9</td>
</tr>
<tr>
<td>Age &gt;60 years</td>
<td>10</td>
<td>31.3</td>
<td>4.1</td>
</tr>
<tr>
<td>Age ≤60 years</td>
<td>22</td>
<td>68.8</td>
<td>10.1</td>
</tr>
<tr>
<td>Smoker</td>
<td>21</td>
<td>65.6</td>
<td>9.1</td>
</tr>
<tr>
<td>Non-smoker</td>
<td>11</td>
<td>34.4</td>
<td>4.9</td>
</tr>
<tr>
<td>Drinker</td>
<td>15</td>
<td>46.9</td>
<td>7.1</td>
</tr>
<tr>
<td>Non-drinker</td>
<td>17</td>
<td>53.1</td>
<td>6.9</td>
</tr>
<tr>
<td>Tumour stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>31.3</td>
<td>3.1</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>25.0</td>
<td>7.1</td>
</tr>
<tr>
<td>3</td>
<td>9</td>
<td>28.1</td>
<td>4.9</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>15.6</td>
<td>0.9</td>
</tr>
<tr>
<td>Recurrence</td>
<td>18</td>
<td>56.25</td>
<td>3.0</td>
</tr>
<tr>
<td>Non-recurrence</td>
<td>14</td>
<td>43.75</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Figure 2. Methylation silences the activity of AKAP12α promoter. AKAP12α transcriptional activity in H1299 cells which had transiently been transfected with the mock-methylated or the methylated AKAP12α promoter constructs is depicted. The mean reporter activity and standard deviation from three independent experiments are presented. pGL3-Basic represents vector backbone without AKAP12α promoter insert.

Methylation status of 5’CpG islands of the AKAP12α gene in lung tissues. In a previous study (13), the methylation of AKAP12α promoter was shown to occur in the two CpG islands and to be associated with lung cancer. For further analysis in the present study, bisulfite-sequencing of CpG islands at AKAP12α promoter was performed in an additional 22 healthy-matched tumour samples. As shown in Figure 3, CpG sites in the AKAP12α promoter were highly methylated in 7 out of 22 tumour samples compared with healthy tissue samples. To investigate the association of the methylation frequencies between healthy and tumour tissues, the paired sample Student’s t-test was carried out for a total of 46 samples, including the 24 samples that were reported in the previous study (13). As shown in Figure 3B, the difference between healthy and tumour tissue samples was statistically significant (p<0.002).

Recently, some reports have shown that promoter methylation of cancer-associated genes is detected in serum DNA of cancer patients (14, 15). In the present study, to investigate whether the methylation of AKAP12α promoter in patient’s serum DNA is matched with that of tissue DNA, comparison analysis was performed in ten paired tissues and serum DNA samples. For each patient, AKAP12α promoter methylation status was analysed in tumour tissue, adjacent normal tissue and preoperative serum DNA. However, there was no correlation in the DNA methylation status between patient tissue and serum samples (Figure 4).

The relationship between AKAP12α promoter methylation status and clinicopathological features of lung cancer patients was analysed. As indicated in Table 1, the CpG island methylation of AKAP12α promoter occurred more frequently in non-relapse patients who were alive without disease for at least two years after curative resection and chemotherapy than in relapse patients within two years after surgical resection. These results suggested that the CpG island methylation of the AKAP12α promoter is associated with longer disease-free duration after curative surgery.

**Discussion**

This study examined whether the methylation of AKAP12α promoter affects AKAP12α gene expression, and also investigated the methylation status of AKAP12α gene in additional lung cancer tissue and serum samples. In agreement with previous results (13), it was found that the expression of the AKAP12α protein is suppressed in human lung cancer cells and is restored after treatment with DNA methyltransferase inhibitor. Using an in vitro methylation assay, it was also shown that the aberrantly methylated promoter of AKAP12α gene induces silencing of AKAP12α gene compared to unmethylated promoter. Furthermore, it was confirmed that promoter methylation of AKAP12α gene occurs more frequently in lung tumour tissues than in adjacent normal tissues. These findings suggest that the methylation status of CpG islands upstream of the AKAP12α promoter may provide a useful indicator to detect silencing of this gene.
in lung cancer, and that the promoter methylation of the AKAP12α gene may be involved in lung carcinogenesis.

The reciprocal relationship between the density of methylated cytosine residues in CpG islands and local transcriptional activity has been widely documented (16). The AKAP12α promoter has been shown to have the characteristics of CpG islands, therefore, suggesting it may be a potential target of inactivation by epigenetic mechanism (4, 17). The previous study showed also that mRNA expression of AKAP12α in lung tumours is silenced by this mechanism (13). Promoter methylation of the AKAP12α gene was detected in lung cancer cells that had lost AKAP12α mRNA expression (13). Furthermore, the role of promoter methylation in the regulation of AKAP12α expression was supported by the present finding that the AKAP12α protein expression is restored by 5-Aza-2-dC treatment, and that transcriptional silencing of AKAP12α is highly induced in methylated AKAP12α promoter compared to unmethylated AKAP12α promoter.

Figure 3. Methylation status of the AKAP12α 5’ CpG islands in lung tissues. A: Representative results of sequencing bisulfite-modified DNA from a matched healthy (N12) and cancer (T12) tissue sample. For each sample, 10 PCR clones were analysed. Fifteen CpG sites are shown with unmethylated (○) and methylated (●) AKAP12α promoter. B: The frequencies of methylation for 46 samples are indicated by the ratio of methylated CpG sites/total CpG sites. Tissue samples: 1-22, additional matched tissue samples; 23-46, samples reported in a previous study (13).

Figure 4. Comparison of AKAP12α promoter methylation status in tissue and serum samples. For each patient, the AKAP12α promoter methylation status was analysed in tumour tissue, adjacent healthy tissue and preoperative serum DNA. The frequencies of methylation for each sample are indicated by the ratio of methylated CpG sites/total CpG sites.
The biological relevance of AKAP12α methylation to lung carcinogenesis has not yet been fully understood, although several mechanisms have been proposed (18, 19). AKAP12α has been associated with cell morphology and cytoskeletal architecture (20), and induces apoptotic cell death in fibrosarcoma by regulating CDK1-cyclin D1 activity; it also functions as a potential metastasis suppressor by inhibiting podosome formation (3). However, its mechanism of action in lung cancer largely remains unclear. Several genes have been shown to be involved in the pathogenesis of lung cancer and frequently inactivated by aberrant promoter methylation (6). The AKAP12α gene is a tumour suppressor gene and its promoter methylation has been shown to cause cancer (4, 17). Nevertheless, the association with AKAP12α methylation and lung cancer remains yet to be determined. In a previous study methylation profiles of the AKAP12α gene were shown in a small set of lung cancer patients (13). In the present study, it was once again demonstrated that AKAP12α promoter is methylated more frequently in lung tumours. Furthermore, the statistical analysis on the additional 22 tissue samples and the previously reported 24 tumour samples showed significant difference in the frequencies of AKAP12α methylation (p<0.002).

In recent years, several studies have reported cell-free tumour specific DNA in serum/plasma of cancer patients (8-10). Furthermore, aberrant methylation of serum/plasma DNA from patients with various types of malignancies has been described (21, 22). In light of these observations, therefore, the methylation status of the AKAP12α gene in matched serum DNA was analysed; however, no significant difference in AKAP12α methylation status was found between lung tumour tissues and matched serum samples. Thus, the results suggested that, although the methylation of AKAP12α promoter is associated with lung cancer, serum DNA is not a useful source of evaluation for the analysis of AKAP12α methylation status.

In summary, the findings of the present study demonstrated the relationship between AKAP12α promoter methylation and lung cancer. Firstly, the methylation was correlated with silencing of AKAP12α protein expression as well as AKAP12α mRNA expression. Secondly, CpG island methylation of the AKAP12α promoter was important in this epigenetic regulation. Further studies aiming to clarify the biological role of the AKAP12α gene may enable AKAP12α to become a DNA methylation-based biomarker of lung cancer.

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References


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