Allelic Loss on Chromosome 3p21.3 and Promoter Hypermethylation of Semaphorin 3B in Non-Small Cell Lung Cancer

Tamotsu Kuroki, Francesco Trapasso, Sai Yendamuri, Ayumi Matsuyama, Hansjuerg Alder, Noel N. Williams, Larry R. Kaiser, and Carlo M. Croce


ABSTRACT

The aim of this study was to evaluate the promoter methylation status and loss of heterozygosity (LOH) of the SEMA3B in non-small cell lung cancers (NSCLCs). We analyzed the methylation status of semaphorin 3B (SEMA3B) promoter and LOH at 3p21.3 in eight NSCLC cell lines and 27 primary tumors. Hypermethylation of SEMA3B was found in 50% of the cell lines and 41% of the primary tumors studied. The presence of hypermethylation was statistically associated with loss of SEMA3B expression in both cell lines (P = 0.02) and primary tumors (P < 0.01). There was no correlation between SEMA3B and tumor stage. On the other hand, the correlation between SEMA3B methylation status and LOH at 3p21.3 was significant (P = 0.02). Notably, 7 of 8 tumors with both hypermethylation and LOH of SEMA3B showed the absence of the expression. Treatment with 5-AZAC restored SEMA3B expression in NSCLC cell line. These results indicate that SEMA3B gene alterations may play an important role in the malignant transformation of NSCLC via a two-hit mechanism, including epigenetic changes and allelic loss, for tumor suppressor gene inactivation.

INTRODUCTION

Allelic loss on chromosome 3p is a frequent event in the multistep carcinogenesis of many types of cancer, including NSCLCs

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Deletion of the region 3p21.3 is one of the most frequent and early event in NSCLC development, indicating that inactivation of tumor suppressor genes on 3p21.3 may play an important role in early steps of NSCLC carcinogenesis (2). Recent studies have suggested that SEMA3B, located at 3p21.3, is a candidate tumor suppressor gene that is frequently inactivated in lung cancer cell lines by promoter hypermethylation (3). Furthermore, introduction and expression of SEMA3B in a human ovarian cancer cell line resulted in suppression of tumor formation (4). SEMA3B is a member of the semaphorin family (5). The semaphorin family of proteins plays a critical role in axonal guidance and contains a highly conserved 500 amino acid semaphorin domain at NH2-terminal (6, 7). Semaphorins have been classified into eight subclasses and five subclasses, classes 3–7 are classified into eight subclasses and five subclasses, classes 3–7 are classified into eight subclasses and five subclasses, classes 3–7 are classified into eight subclasses and five subclasses, classes 3–7 are classified into eight subclasses and five subclasses. Semaphorins 3B, which mediate axon guidance and cell migration, are the most thoroughly characterized members of the semaphorin family. Semaphorin 3B has been shown to play a potential tumor suppressor role in tumorigenesis (3, 5, 9). In this study, we analyzed the promoter methylation status of SEMA3B in NSCLCs by using MSP. We also examined these samples for LOH at the SEMA3B locus by analysis of microsatellite markers flanking 3p21.3. The frequency and potential clinical implications of hypermethylation of SEMA3B and the correlation between methylation status and LOH at 3p21.3 in NSCLCs were investigated in this study.

MATERIALS AND METHODS

Cell Lines and Tissues. Human lung cancer cell lines Calu-3, A549, NCI-H23, NCI-H460, NCI-H522, NCI-H1299, NCI-H1573, and NCI-H1650 were obtained from the American Type Culture Collection. All cell lines were maintained in RPMI 1640 with 10% fetal bovine serum at 37°C and 5% CO2. The tumor samples and their corresponding noncancerous tissues were obtained from 27 patients who underwent surgery for NSCLC at the Hospital of the University of Pennsylvania (18 male and 9 female; median age, 67.6 years; range, 51–80 years). Of these 27 samples, 11 were squamous cell carcinomas, 10 were adenocarcinomas, 4 were poorly differentiated carcinomas, and 2 samples had other histologies. Seventeen of them were stage I, 7 were stage II, and 3 were stage III, according to the TNM classification.

DNA and RNA Extraction. The tissue samples were excised and immediately stored at −80°C. DNA and RNA were extracted from each of the eight cell lines and from the 27 paired lung tissues according to methods described previously (10).

LOH Analysis. Allelic losses were analyzed by a PCR approach with primers amplifying polymorphic microsatellites flanking the SEMA3B gene at loci D3S1568, D3S1621, and D3S84597. The primer sequences were obtained from the Genome Database, and primers were labeled using 5'-fluorescein phosphoramidite for microsatellite loci, as described by Ishii et al. (11). PCR was performed on the genomic DNA samples using the following conditions: 50 ng of genomic DNA template, 10 pmol of each primer, 2.5 mM MgCl2, 1.5 mM deoxynucleoside triphosphate mix, 1× PCR buffer, and 0.5 unit of AmpliTaq Gold (Perkin-Elmer, Branchburg, NJ) in a 20-μl final volume. PCR cycles included one cycle of 95°C for 12 min followed by 35 cycles consisting of 10 cycles at 94°C for 15 s, 55°C for 15 s, and 72°C for 30 s and 25 cycles at 89°C for 15 s, 55°C for 15 s, and 72°C for 30 s, followed by 72°C for 10 min in a Perkin-Elmer Gene Amp PCR system 9600. PCR products were denatured in formamide for 5 min at 95°C and then loaded on a 6% denaturing gel on the Applied Biosystems 373 DNA sequencer. LOH was analyzed by using the Applied Biosystems Prism Genescan and the Applied Biosystems Prism Genetyper Analysis software (Perkin-Elmer/Applied Biosystems). Cases were defined as LOH when an allele peak signal from tumor DNA was reduced by 50% compared with the normal counterpart.

Methylation Analysis. The methylation status in the promoter region of the SEMA3B was determined by MSP as described previously (12). DNA methylation patterns in the promoter region of the SEMA3B gene were determined by sequencing bisulfite-treated DNA (3). Primer sequences for methylation analysis of SEMA3B promoter region were for the unmethylated reaction (sense) and for the methylated reaction (antisense) and for the methylated reaction (antisense) and for the methylated reaction (antisense) and for the methylated reaction (antisense). 5'-ATCAAATAAAAGAACAAAACAC-3' (antisense) and for the methylated reaction (antisense). 5'-ATCAAATAAAAGAACAAAACAC-3' (antisense). Briefly, 1 μg of the genomic DNA was denatured by 2 μl NaOH and then incubated in 3 μl sodium bisulfite and 10 mM hydroquinone for 16 h at 55°C. Bisulfite-treated DNA was extracted using a genomic DNA clean-up kit (Promega, Madison, WI). Modified DNA was amplified by two different set of primers specific for unmethylated and methylated SEMA3B promoter region sequences. Human genomic DNA (Clontech, Palo Alto, CA) treated in vitro with SsI methylase (New England Biolabs, Inc., Beverly, MA) was used as a positive control. PCR was performed in 50-μl reaction volumes containing 1× PCR buffer II (Perkin-Elmer, Branchburg, NJ), 2 mM MgCl2, 0.2 mM of each deoxynucleoside

Received 12/19/02; accepted 4/15/03.

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3 The abbreviations used are: NSCLC, non-small cell lung cancer; SEMA3B, semaphorin 3B; MSP, methylation-specific PCR; LOH, loss of heterozygosity; TNM, Tumor-Node-Metastasis; RT-PCR, reverse transcription-PCR; 5-AZAC, 5-aza-2'-deoxycytidine.

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RESULTS

Hypermethylation Analysis of SEMA3B. To evaluate the methylation status of the promoter region of SEMA3B in NSCLCs, we used a MSP approach. Four of eight (50%) NSCLC cell lines (NCI-H23, NCI-H1299, NCI-H1573, and NCI-H1650) showed methylated bands of SEMA3B, and five (63%) cell lines (Calu-3, A549, NCI-H460, NCI-H522, and NCI-H1650) showed unmethylated bands. Both methylated and unmethylated bands were detected in one (13%) cell line (NCI-H1650; Fig. 1A). Hypermethylation of the promoter region of SEMA3B was detected in 11 of 27 (41%) NSCLCs. Unmethylated bands were detected in all 27 tumor samples. In primary tumors, the presence of the remaining stromal cells may contribute to the presence of unmethylated bands in all cases. Unmethylated bands were detected in all of the corresponding noncancerous tissues. Three of 27 noncancerous tissues (11%) showed methylated bands. Representative MSP results of NSCLCs are shown in Fig. 1B. The results of statistical analysis for correlation of SEMA3B promoter methylation status in the tumor tissues with clinicopathological data are shown in Table 1. There were no significant correlations of SEMA3B promoter hypermethylation with gender, age, histological subtype, T stage, N stage, and TNM classification stage.

LOH Analysis at 3p21.3. LOH in SEMA3B region at 3p21.3 was examined in 27 NSCLCs using three microsatellite markers. LOH was detected in 12 (44%) of 27 tumors that were informative for at least one of the three markers at 3p21.3 for the SEMA3B region. We also examined the relationship between methylation status and the presence of LOH. Among 27 informative cases for SEMA3B region, 8 (67%) of 12 tumors with LOH showed hypermethylation of SEMA3B, and 3 (20%) of 15 tumors without LOH showed hypermethylation of SEMA3B. The correlation between SEMA3B hypermethylation and LOH at SEMA3B region was statistically significant (Fisher’s exact test, P = 0.02).

RT-PCR Analysis of SEMA3B. The mRNA expression of SEMA3B was analyzed in eight NSCLC cell lines by RT-PCR. Four of eight cell lines (Calu-3, A549, NCI-H460, NCI-H522) expressed the SEMA3B mRNA (Fig. 2A). In all four cell lines (Calu-3, A549, NCI-H460, NCI-H522), SEMA3B was expressed in the absence of methylated SEMA3B allele. In one cell line (NCI-H1650), SEMA3B was not expressed in the presence of both methylated and unmethylated alleles. In three cell lines (NCI-H23, NCI-H1299, NCI-H1573) that showed only methylated alleles, SEMA3B was not expressed. Thus, there was a statistically significant association between SEMA3B promoter hypermethylation and SEMA3B expression in cell lines (chi-square test, P = 0.02). SEMA3B expression was examined by RT-PCR in 27 primary NSCLCs and corresponding noncancerous tissues. RT-PCR analysis demonstrated that no SEMA3B transcripts were amplified from seven tumor tissues. All these seven tumors demonstrated the SEMA3B promoter hypermethylation. The remaining 20 tumors and all 27 noncancerous tissues showed SEMA3B expression. In 16 of 20 tumors, SEMA3B was expressed with the nonhypermethylation of the SEMA3B promoter. Overall, there was a

Table 1: Clinicopathological correlation of SEMA3B promoter hypermethylation

<table>
<thead>
<tr>
<th>Gender</th>
<th>Hypermethylation</th>
<th>Nonhypermethylation</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>8</td>
<td>10</td>
<td>0.58a</td>
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<tr>
<td>Female</td>
<td>3</td>
<td>6</td>
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Note: *Fisher’s exact test.
**Student’s t test.
***Chi-square test.

Triphosphate, 10 pmol of each primer, 1 unit of AmpliTaq Gold polymerase (Perkin-Elmer), and 50 ng of bisulfite-modified DNA. PCR cycling conditions were as follows: 10 min at 95°C with a hot start; 40 cycles of a 30 s denaturation at 94°C, a 30-s annealing at 58°C; a 30-s extension at 72°C; and a final extension step of 5 min at 72°C.

PCR products were analyzed on 2% agarose gels with ethidium bromide and visualized under UV illumination. To verify the methylation status determined by MSP, both unmethylated and methylated products were excised from the gel, purified using the QiAquick gel extraction kit (Qiagen), and sequenced on the Applied Biosystems Prism 377 DNA sequencing system.

RT-PCR Analysis of the SEMA3B Transcript. cDNAs were synthesized from 2 μg of total RNA, and the reverse transcription was performed as described previously (13). Glyceraldehyde-3-phosphate dehydrogenase amplification from the same cDNA samples was used for an internal control. One μl of cDNA was used for PCR amplification with SEMA3B-specific primers (sense primer, 5′-TTCTTTCGTGAGACGGCGGTA-3′; antisense primer, 5′-CCCTGGAAGATGCTGCTGGA-3′) in a volume of 50 μl containing 20 pmol of each primer, 2.5 mm MgCl₂, 1.5 mm deoxynucleoside triphosphate mix, 1× PCR buffer, and 2 units of AmpliTaq Gold (Perkin-Elmer). PCR cycles included one cycle of 95°C for 5 min followed by 40 cycles each of 45°C for 30 s, 58°C for 30 s, and 72°C for 30 s and a final extension step of 72°C for 5 min in a Perkin-Elmer Gene Amp PCR system 9600. The amplified products were analyzed by electrophoresis on a 2% agarose gel. DNA bands of SEMA3B transcripts were excised from the gel, purified using the QiAquick gel extraction kit (Qiagen, Valencia, CA), and sequenced on the Applied Biosystems Prism 377 DNA sequencing system (Perkin-Elmer Corp., Foster City, CA).

5-AZAC Treatment. To determine whether treatment of nonexpressing cell line with a demethylating agent induced SEMA3B expression, we treated the NSCLC cell line NCI-H23 (not expressing SEMA3B) with 5-AZAC. Cells were plated and incubated for 3 days with 1 μM 5-AZAC. The medium and the drug were replaced every 24 h.

Statistical Analysis. Statistical analysis was performed using the Fisher’s exact test, Student’s t test, and the chi-square test; P < 0.05 were regarded as statistically significant.

LOH AT 3P AND HYPERMETHYLATION OF SEMA3B IN LUNG CANCER

<table>
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<th>Nonhypermethylation</th>
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<tbody>
<tr>
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<td>0.96'</td>
</tr>
<tr>
<td>T2</td>
<td>7</td>
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<td>T3</td>
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</tr>
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Stage

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</tr>
<tr>
<td>II</td>
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<td>5</td>
</tr>
<tr>
<td>III</td>
<td>2</td>
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</table>

Promoter hypermethylation alleles. In three cell lines (NCI-H23, NCI-H1299, NCI-H1573) was not expressed in the presence of both methylated and unmethylated alleles. In all four cell lines (Calu-3, A549, NCI-H460, NCI-H522) showed unmethylated bands. Both methylated and unmethylated bands were detected in one (13%) cell line (NCI-H1650; Fig. 1A). Hypermethylation of the promoter region of SEMA3B was detected in 11 of 27 (41%) NSCLCs. Unmethylated bands were detected in all 27 tumor samples. In primary tumors, the presence of the remaining stromal cells may contribute to the presence of unmethylated bands in all cases. Unmethylated bands were detected in all of the corresponding noncancerous tissues. Three of 27 noncancerous tissues (11%) showed methylated bands. Representative MSP results of NSCLCs are shown in Fig. 1B. The results of statistical analysis for correlation of SEMA3B promoter methylation status in the tumor tissues with clinicopathological data are shown in Table 1. There were no significant correlations of SEMA3B promoter hypermethylation with gender, age, histological subtype, T stage, N stage, and TNM classification stage.

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significant correlation between SEMA3B promoter hypermethylation and loss of SEMA3B expression in clinical samples (Fisher’s exact test, P < 0.01). Representative RT-PCR results of primary NSCLCs are shown in Fig. 2B. In addition, we investigated the patterns of SEMA3B expression, SEMA3B promoter methylation status, and LOH at the SEMA3B locus. Interestingly, among 11 tumors that exhibited hypermethylation, 7 with LOH showed the absence of the expression of SEMA3B. All 7 tumors that exhibited the loss of SEMA3B expression showed a hypermethylation with LOH (Table 2). There was a significant correlation between the SEMA3B expression and the pattern of SEMA3B status, including hypermethylation and LOH (P < 0.01).

5-AZAC Treatment. Lung cancer cell line NCI-H23 that showed complete hypermethylation and loss of expression of SEMA3B was cultured with the demethylating agent 5-AZAC. 5-AZAC treatment restored SEMA3B expression in NCI-H23 cell line (Fig. 2C).

**DISCUSSION**

Recent studies have reported that biallelic inactivation of several tumor suppressor genes, including fragile histidine triad, retinoic acid receptor-β, and RASSF1A by allelic loss and hypermethylation of the promoter region, is a common event in human cancers (14–16). In this study, we demonstrated that SEMA3B hypermethylation significantly correlated with LOH of SEMA3B locus at 3p21.3 in NSCLCs. Furthermore, 7 of 8 (88%) tumor samples with both hypermethylation and LOH of SEMA3B showed the absence of the SEMA3B expression. Previous studies have demonstrated that mutations are infrequent in SEMA3B (5). Sekido et al. (5) reported that loss of SEMA3B mRNA expression was detected in 44% of NSCLC cell lines. In this study, four NSCLC cell lines (50%) showed the absence of SEMA3B mRNA expression, and SEMA3B hypermethylation was observed in at least one allele in all these cell lines. These findings suggest that SEMA3B gene alterations may play a role in the tumorigenesis of NSCLC via a two-hit mechanism, including epigenetic changes and allelic loss for tumor suppressor gene inactivation (17). On the other hand, treatment with the demethylating drug 5-AZAC restored SEMA3B expression in NSCLC cell line. This suggests that SEMA3B promoter hypermethylation is responsible for silencing SEMA3B expression in NSCLC cell line. In this study, SEMA3B promoter hypermethylation of noncancerous tissues was detected in 11% (3 of 27) patients with NSCLCs. In addition, this study showed that the SEMA3B promoter region was methylated not only in advanced stage tumors but also in early stage of NSCLCs. These findings suggest that SEMA3B promoter hypermethylation could be an early event in the pathogenesis of some cases of NSCLCs. Furthermore, the presence of the hypermethylation in corresponding noncancerous tissues may represent the appearance of precancerous lesions. Recently, Lee et al. (18) reported that promoter hypermethylation of several genes can be detected in the serum of gastric cancer patients using MSP. Thus, detection of SEMA3B using MSP could be a useful method for the early detection and diagnosis in the screening of NSCLCs. Another class 3 semaphorin, SEMA3A, has been shown to induce neuronal apoptosis (19). On the other hand, recent studies have shown that SEMA3B inhibits the growth of lung cancer cell lines through the induction of apoptosis (3). Interestingly, Ochi et al. (20) reported that SEMA3B is a direct transcriptional target of p53 and might involve p53-dependent apoptosis in cancer cells. In conclusion, two-hit gene silencing of SEMA3B through epigenetic change and allele loss is a common and important event in the carcinogenesis of NSCLCs. Thus, additional studies are called for to elucidate the mechanistic role of SEMA3B in the pathogenesis of NSCLC.

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