Allele Loss and Promoter Hypermethylation of VHL, RAR-β, RASSF1A, and FHIT Tumor Suppressor Genes on Chromosome 3p in Esophageal Squamous Cell Carcinoma

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ABSTRACT

Promoter hypermethylation is an alternative way to inactivate tumor suppressor genes in cancer. Alterations of chromosome 3p are frequently involved in many types of cancer, including esophageal squamous cell carcinoma. Here, we investigated the methylation status and loss of heterozygosity (LOH) of 3p tumor suppressor genes. We examined the promoter methylation status of von Hippel-Lindau disease (VHL), retinoic acid receptor β (RAR-β), RAS association domain family 1A (RASSF1A), and fragile histidine triad (FHIT) genes in 22 esophageal squamous cell carcinoma cell lines and 47 primary tumors and corresponding noncancerous tissues by a methylation-specific PCR. In addition, we analyzed 47 paired samples for LOH at eight loci on chromosome 3p. Hypermethylation in VHL, RAR-β, RASSF1A, and FHIT was detected in 36, 73, 73, and 50% of tumor cell lines, respectively. In primary tumors, hypermethylation in VHL, RAR-β, RASSF1A, and FHIT was detected in 13, 55, 51, and 45%, respectively. In corresponding noncancerous tissues, hypermethylation in RAR-β and FHIT was frequently detected in 38 and 30%, respectively, whereas no VHL hypermethylation and only 4% of RASSF1A hypermethylation were detected. Furthermore, in clinical stages I and II, hypermethylation in RAR-β (67%) and FHIT (78%) was frequently detected, whereas no VHL hypermethylation and 11% of RASSF1A hypermethylation were detected. On the other hand, the correlation between FHIT hypermethylation and LOH at FHIT region was statistically significant (P = 0.008). Our findings suggest that hypermethylation of the RAR-β and FHIT may play an important role in the early stage of esophageal squamous cell carcinogenesis. In addition, FHIT may be inactivated in accordance with the two-hit inactivation model, involving deletion of one allele and hypermethylation of the other.

INTRODUCTION

The development of human cancer is generally thought to be a multistep process that involves multiple genetic and epigenetic changes. Recently, several studies have shown that methylation of CpG islands located within the promoter regions of tumor suppressor genes is a frequent event in the development of several human malignancies (1–5). This modification, a well-known epigenetic change, has been proposed to be an alternative way of inactivation of tumor suppressor genes in cancer (1–5). In esophageal squamous cell carcinomas, hypermethylation of the FHIT gene promoter has been reported to be associated with transcriptional inactivation (6).

Chromosome 3p allelic losses are frequent events in many types of cancer including esophageal squamous cell carcinoma, suggesting the presence of multiple tumor suppressor genes on 3p (7, 8). Several 3p tumor suppressor genes have been identified, including VHL3 at 3p25, RAR-β at 3p24, RASSF1A at 3p21.3, and FHIT at 3p14.2, on chromosome 3p (9–12). In the present study, we have analyzed the promoter methylation status of the tumor suppressor genes on 3p including VHL, RAR-β, RASSF1A, and FHIT in esophageal squamous cell carcinomas using MSP. We also examined these samples for LOH at tumor suppressor gene regions of 3p. The frequency and the potential clinical implications of hypermethylation of multiple 3p tumor suppressor genes and the correlation between methylation status and LOH at tumor suppressor gene regions of 3p in human esophageal squamous cell carcinomas have been investigated.

MATERIALS AND METHODS

Cell Lines and Tissues. Twenty-two human esophageal carcinoma cell lines were used; the TE series (13) was provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University, Japan; the KYSE series (9) was a generous gift from Dr. Shimada (Kyoto University, Kyoto, Japan). The primary tumor samples and their corresponding noncancerous tissues were obtained from 47 Japanese patients who underwent surgery for esophageal squamous cell carcinoma (44 male and 3 female; median age, 61 years; range, 48–74 years). Four of them were stage I, 5 were stage II, 36 were stage III, and 2 were stage IV, according to the Tumor-Node-Metastasis 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 22 cell lines, and from the 47 paired esophageal tissues according to methods described previously (13).

Methylation Analysis. The methylation status in the promoter region of VHL (14), RAR-β (15), RASSF1A (16), and FHIT (17) was determined by MSP as described previously. The primer sequences of each gene, annealing temperatures, and the expected product size are listed in Table 1. Briefly, 1 μg of the genomic DNA was denatured by 2 M NaOH and then was incubated in 3 M sodium bisulfite and 10 mM hydroquinone for 17 h at 55°C. Bisulfite-treated DNA was extracted using a genomic DNA clean-up kit (Promega, Madison, WI). Modified DNA was amplified by specific primers for both unmethylated and methylated sequences. PCR was performed in 50-μl reaction volumes containing 1× PCR buffer II (Perkin-Elmer, Branchburg, NJ), 2 mM MgCl2, 0.2 mM each deoxynucleoside 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, 40 cycles of 30 s denaturation at 94°C, 30 s at a specific annealing temperature, a 30 s extension at 72°C, and a final extension step of 5 min at 72°C. Human genomic DNA (Clontech, Palo Alto, CA) treated in vitro with SssI methylase (New England Biolabs, Inc., Beverly, MA) was used as a positive control. PCR products were analyzed on 2% or 3% 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.
LOH Analysis. Paired normal and tumor DNA samples were analyzed with eight polymorphic microsatellite markers. The locations of these markers and the locations of the VHL, RAR-β, RASSF1A, and FHIT are listed in Table 5. All of the microsatellite markers used in this analysis were CA-repeat markers: D3S1038 and D3S1317 for VHL; D3S598 and D3S1266 for RAR-β; D3S1568 and D3S1621 for RASSF1A; D3S1234 and D3S1300 for FHIT. The primer sequences were obtained from the Genome Database, and primers were labeled using 5’-fluorescein phosphoramidite or 5’-tetrachlorofluorescein phosphoramidite for microsatellite loci, as described by Ishii et al. (18). 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 dNTP mix, 1× PCR buffer, and 0.5 unit of AmpliTaq Gold (Perkin-Elmer) 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 95°C for 15 s, 72°C for 15 s, and 72°C for 1 min, and a final extension step of 72°C for 5 min in a Perkin-Elmer Gene Amp PCR system 9600. PCR products were denatured in formamide for 5 min at 95°C and then were 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.

RT-PCR Analysis. cDNAs were synthesized from 2 µg of total RNA, and the RT was performed as described previously (13). GAPDH amplification was used for cDNA normalization. The locations of primers used for RT-PCR analyses were used according to previous reports (19–21). One µl of cDNA was used for PCR amplification with each specific primer in a volume of 50 µl containing 20 pmol of each primer, 2.5 mM MgCl₂, 1.5 mM dNTP mix, 1× PCR buffer, and 2 units of AmpliTaq Gold (Perkin-Elmer). PCR cycles included 1 cycle of 95°C for 10 min followed by 35 cycles each of 94°C for 30 s, specific annealing temperature for 30 s, and 72°C for 1 min, and a final extension step at 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 each transcript 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).

Statistical Analysis. Fisher’s exact test and Student’s t test were used for statistical analysis; Ps less than 0.05 were regarded as statistically significant.

RESULTS

Methylation Analysis. We determined the frequency of methylation of VHL, RAR-β, RASSF1A, and FHIT in 22 esophageal carcinoma cell lines and 47 primary esophageal squamous cell carcinomas with their corresponding noncancerous tissues by MSP. The primers used in this study are listed in Table 1. Fig. 1 summarizes the frequency of the methylated and unmethylated alleles of each gene in 22 cell lines. Among twenty-two cell lines, methylated bands were detected in 8 (36%) of 22 for VHL, 16 (73%) for RAR-β, 16 (73%) for RASSF1A, and 11 (50%) for FHIT. Unmethylated bands were detected in 20 (91%) of 22 for VHL, 14 (64%) for RAR-β, 9 (41%) for RASSF1A, and 13 (59%) for FHIT. Both methylated and unmethylated bands were detected in 6 (27%) of 22 for VHL, 8 (36%) for RAR-β, 3 (14%) for RASSF1A, and 2 (9%) for FHIT.

In the primary tumor samples, frequent hypermethylation was detected in RAR-β (55%), RASSF1A (51%), and FHIT (45%). By contrast, hypermethylation of VHL was detected in only 13%. In the corresponding noncancerous tissues, hypermethylation was frequently detected in RAR-β (38%) and FHIT (30%); however, hypermethylation of RASSF1A (4%) and VHL (0%) was rare. The frequency of methylation of the four genes in 47 primary esophageal squamous cell carcinomas and their corresponding noncancerous tissues are detailed in Table 2. Unmethylated bands were detected in 47 (100%) of 47 for

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**Table 1. Primer sequences for MSP**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequencea</th>
<th>Annealing temperature (°C)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VHL</td>
<td>U: GTTGGAGGATTTTGTGTATGT (sense)</td>
<td>60</td>
<td>165</td>
</tr>
<tr>
<td></td>
<td>M: CCACAACCAACACAACACA (antisense)</td>
<td>60</td>
<td>165</td>
</tr>
<tr>
<td></td>
<td>M: GAGCGAACGCGCCTGAC (sense)</td>
<td>60</td>
<td>165</td>
</tr>
<tr>
<td>RAR-β</td>
<td>U: TTGAGAATGTGATGTTTGA (sense)</td>
<td>55</td>
<td>146</td>
</tr>
<tr>
<td></td>
<td>M: CAGATAACCTACACTCAACCAA (antisense)</td>
<td>55</td>
<td>146</td>
</tr>
<tr>
<td></td>
<td>M: TTGGTTTCTGGGGAAGATCG (sense)</td>
<td>55</td>
<td>146</td>
</tr>
<tr>
<td>RASSF1A</td>
<td>U: GGTTTTGGATTTTGTGTATGTAT (sense)</td>
<td>55</td>
<td>146</td>
</tr>
<tr>
<td></td>
<td>M: CTAACACAAAAAACAACCAACCAA (antisense)</td>
<td>55</td>
<td>146</td>
</tr>
<tr>
<td></td>
<td>M: TDGGGTTTCTGGGGAAGATCG (sense)</td>
<td>55</td>
<td>146</td>
</tr>
<tr>
<td>FHIT</td>
<td>U: TTGGGTTTCTGGGGAAGATCG (sense)</td>
<td>66</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>M: TDGGGTTTCTGGGGAAGATCG (sense)</td>
<td>66</td>
<td>74</td>
</tr>
</tbody>
</table>

a U, unmethylated; M, methylated.

**Table 2. The frequency of promoter hypermethylation of VHL, RAR-β, RASSF1A, and FHIT in esophageal squamous cell carcinomas and corresponding nonmalignant esophageal tissues (n = 47)**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Tumor (%)</th>
<th>Nonmalignant tissue (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VHL</td>
<td>6 (13)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>RAR-β</td>
<td>26 (55)</td>
<td>18 (38)</td>
</tr>
<tr>
<td>RASSF1A</td>
<td>24 (51)</td>
<td>2 (4)</td>
</tr>
<tr>
<td>FHIT</td>
<td>21 (45)</td>
<td>14 (30)</td>
</tr>
</tbody>
</table>

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**Fig. 2.** Representative results of MSP analysis for four genes. MSP analysis of each gene promoter by using both unmethylated (U) and methylated (M) specific primers. PC, positive control; NC, negative control; T, tumor; N, corresponding normal tissue. Arrow, product size.
VHL, 42 (89%) for RAR-β, 36 (77%) for RASSF1A, and 46 (98%) for FHIT in tumor samples. However, in primary tumors, the presence of the remaining stromal cells may contribute to the presence of unmethylated bands in many cases. Unmethylated bands of all four genes were detected in all 47 of the corresponding noncancerous tissues. The PCR products obtained with the unmethylated and methylated primers of three different tumors and corresponding noncancerous tissues were directly sequenced. In the PCR product from unmethylated primers, all of the unmethylated cytosine nucleotides including those in the CpG dinucleotides changed to thymines as a result of bisulfite modification. Cytosine nucleotides in the CpG dinucleotides were found to remain as cytosine, which indicated the presence of methylated DNA (data not shown). Representative results of MSP for four genes are shown in Fig. 2. The results of hypermethylation for each gene in all of the tumors with their corresponding noncancerous tissues are shown in Fig. 3.

Methylation and Clinicopathological Correlations. The results of statistical analysis for correlation of promoter methylation status of different 3p genes in the tumor tissues with histological differentiation and tumor stage are shown in Table 3. There were no significant correlations of promoter hypermethylation with age and histological differentiation of all genes. However, there was a significant correlation between RASSF1A promoter hypermethylation and tumor stage ($P = 0.01$; Fisher’s exact test). In clinical stages I and II, frequencies of hypermethylation were detected 0 (0%) of 9 for VHL, and 1 (11%) for RASSF1A; in contrast, hypermethylation were detected 6 (67%) for RAR-β, and 7 (78%) for FHIT in tumor samples.

Concurrent hypermethylation in multiple genes was analyzed. Forty-two (89%) tumors showed hypermethylation in at least one of the four genes examined. Concurrent hypermethylation in three or four genes was more common in clinical stages III and IV (9 of 38; 24%) than in stages I and II (0 of 9; 0%). However, there was no significant correlation between concurrent hypermethylation and clinical stage (Table 4).

Methylation and LOH Correlations. LOH of four different tumor suppressor gene regions on chromosome 3 were examined in 47 esophageal squamous cell carcinomas using eight microsatellite markers (Table 5). LOH was detected in 12 (27%) of 45 tumors that were informative for at least one of the two markers on 3p25 for VHL.

![Fig. 3. Summary of hypermethylation for four genes. White boxes, samples that are not methylated in both tumor and corresponding normal tissue; red boxes, samples that are methylated in only tumor; green boxes, samples that are methylated in both tumor and corresponding normal tissue.](image.png)
region, 24 (56%) of 43 for RAR-β, 23 (55%) of 42 for RASSF1A, and 24 (55%) of 44 for FHIT (Table 5). In 36 (77%) tumors, LOH was detected at one or more 3p markers. The detailed results of LOH analysis for each marker in all of the tumors are shown in Fig. 4. We also examined the relationship between methylation status and the presence of LOH on each region of 3p tumor suppressor genes (Table 6). Among 44 informative cases for FHIT region, 15 (65%) of 23 tumors with LOH showed hypermethylation of FHIT, and 5 (24%) of 21 tumors without LOH showed hypermethylation of FHIT. The correlation between FHIT hypermethylation and LOH at FHIT region was statistically significant (P = 0.008). However, there was no correlation between hypermethylation of VHL, RAR-β, or RASSF1A and LOH at each region of 3p tumor suppressor genes.

**RT-PCR Analysis.** We analyzed three genes (RAR-β, RASSF1A, and FHIT) expression that showed frequent hypermethylation in 19 primary esophageal squamous cell carcinomas and corresponding noncancerous tissues. RT-PCR analysis demonstrated that no transcripts were amplified from 6 (32%) in RAR-β, 5 (26%) in RASSF1A, or 5 (26%) in FHIT of tumor tissues, respectively. Representative RT-PCR results are shown in Fig. 5. In addition, we investigated the patterns of these genes expression, promoter methylation status, and LOH at each locus. Interestingly, among the no-transcripts cases, hypermethylation and/or LOH was detected in 83% (5 of 6) for RAR-β, in 100% (5 of 5) for RASSF1A, and in 100% (5 of 5) for FHIT (Table 7). However, there were no significant correlations between the expression and the pattern of hypermethylation and LOH for each gene.

**DISCUSSION**

We have described the methylation status of the promoter region of multiple tumor suppressor genes at chromosome 3p in esophageal squamous cell carcinomas and its relationship with clinicopathological factors. Furthermore, we analyzed the correlation between hypermethylation and LOH of the tumor suppressor genes at each region. Our results showed high hypermethylation frequency for RAR-β, RASSF1A, and FHIT in tumor tissue. On the other hand, in the corresponding noncancerous tissues, we also found high hypermethylation frequency for RAR-β and FHIT. Interestingly, the present study showed that RAR-β and FHIT were methylated not only in advanced-stage tumors but also in early stages of development of esophageal squamous cell carcinoma. Recent studies indicated RAR-β (22) and FHIT (17) hypermethylation in a few samples of corresponding normal colon, breast, and lung tissues associated with cancers. Our previous study has shown that a loss of FHIT expression was already
detectable in precarcinomatous lesions of the esophagus by immunostaining (23). These findings suggest that RAR-β and FHIT hypermethylation may play an important role in the early steps of tumor progression of esophageal squamous cell carcinoma. Furthermore, the presence of the hypermethylation of these genes in nonmalignant tissues may represent the appearance of premalignant lesions. Thus, detection of RAR-β and FHIT hypermethylation using MSP may provide potential new molecular diagnostic markers of esophageal squamous cell carcinomas at an early stage during multistep carcinogenesis. Laird et al. (24) demonstrated that the inhibition of DNA methylation can suppress tumor initiation of intestinal neoplasia in a tumor-induced mouse model. Thus, the inhibition of RAR-β and/or FHIT hypermethylation may be a useful approach as a preventive strategy. In the present study, concurrent hypermethylation in three or four tumor suppressor genes was detected only in advanced stages of the disease. This finding suggests that the accumulation of hypermethylation of tumor suppressor genes contributes to the tumor progression during tumor development. Furthermore, methylation analysis of multiple tumor suppressor genes at 3p may be a useful tool for providing prognostic marker in esophageal squamous cell carcinomas.

Previous work has shown that LOH at the FHIT gene region was observed frequently in esophageal squamous cell carcinomas (23). The present study demonstrated that FHIT hypermethylation significantly correlated with LOH at FHIT region in esophageal squamous cell carcinomas. These results suggest that FHIT gene alterations may play a role in the esophageal squamous cell carcinogenesis via a two-hit mechanism as proposed by Knudson (25), including epigenetic changes for tumor suppressor gene inactivation.

Our findings indicate that an analysis of the methylation status of the tumor suppressor genes along with an LOH analysis may lead to approaches that may contribute to an improved outcome for patients with esophageal squamous cell carcinoma.

REFERENCES

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