Hypermethylation-Associated Inactivation of Retinoic Acid Receptor β in Human Esophageal Squamous Cell Carcinoma

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ABSTRACT

Purpose: The purpose of this study was to investigate the mechanism of altered retinoic acid receptor β (RARβ) expression during esophageal squamous carcinogenesis.

Experimental Design: Samples were collected from Linzhou, China. The hypermethylation of CpG islands in the promoter region of the RARβ gene was examined by methylation-specific PCR in human esophageal squamous cell carcinoma (ESCC) samples, as well as in neighboring tissues with normal epithelium, basal cell hyperplasia, and dysplasia. RARβ mRNA expression was determined by in situ hybridization. The DNA methyltransferase inhibitor 2′-deoxy-5-azacytidine was used to treat the ESCC cell line, and the DNA hypermethylation status and mRNA expression level were examined.

Results: Two of 17 (12%) normal, 9 of 21 basal cell hyperplasia (43%), 7 of 12 dysplasia (58%), and 14 of 20 ESCC (70%) samples had hypermethylation of the RARβ promoter region. The loss of RARβ mRNA expression was highly concordant with RARβ promoter CpG island hypermethylation when individual samples were considered in the correlation analysis. Good statistical correlation between hypermethylation and loss of RARβ expression was revealed. Frequencies of hypermethylation appeared to increase with the progression of carcinogenesis. In samples from the same patients, if hypermethylation was detected in earlier lesions, it was usually observed in more severe lesions. In the ESCC cell line KYSE 510, 2′-deoxy-5-azacytidine partially reversed CpG island hypermethylation and restored RARβ mRNA expression.

Conclusions: The results suggest that hypermethylation of RARβ promoter region is an important mechanism for RARβ gene silencing in esophageal squamous carcinogenesis.

INTRODUCTION

Retinoic acid and its analogues can modulate cell growth and differentiation in vitro and in vivo (1, 2). Retinoids have been used successfully in the treatment of preneoplastic diseases, such as oral leukoplakia (3), cervical dysplasia (4), and xeroderma pigmentosum (5). These compounds have also been reported to reduce second malignancies in the liver, aerodigestive tract, and breast (6–9). Retinoids exert their biological effects by binding to the specific nuclear retinoid receptors RARs9 and retinoid X receptors, each of which consists of three subtypes (α, β, and γ). RARs are differentially expressed during development and in adult life, and there is strong evidence that RARβ plays a central role in growth regulation of epithelial cells and in tumorigenesis (10–12). RARβ and retinoid X receptor-dependent pathways, but not RARα- or RARγ-dependent pathways, lead to cyclin D1 degradation and blockage of cell cycle progression (13).

RARβ expression is frequently down-regulated in human cancers, including those of the head and neck, lung, breast, pancreas, and esophagus (14). In a study of esophageal cancer, RARβ expression was lost in 54% of ESCC and 57% of dysplastic lesions (15). RARβ loss of expression was not correlated with loss of heterozygosity at the RARβ chromosome location 3p24 in esophageal cancer (16). The molecular mechanism for down-regulation or loss of RARβ expression in ESCC is still unknown.

Hypermethylation of CpG islands is an important epigenetic mechanism for the transcriptional silencing of many genes (17–19). DNA hypermethylation may directly affect the basal transcriptional machinery by altering the DNA secondary structure and inducing chromosome remodeling through the methyl-group-binding proteins and histone deacetylase, which leads to transcriptional repression (20). Treatment with DAC, a DNA methyltransferase inhibitor, causes the reactivation of many genes that have been inactivated by hypermethylation (21–25). Hypermethylation of CpG islands in the RARβ promoter region has been reported in many human cancers, including breast, lung, colon, stomach, cervical, bladder, and prostate cancers (26–31). In lung and breast cancers, hypermethylation of the RARβ promoter region was found to be the mechanism for...
Hypermethylation of RARβ in ESCC

Table 1  Primer sequences, annealing temperatures, and PCR product sizes used for MSP and reverse transcription-PCR

<table>
<thead>
<tr>
<th>Target locus</th>
<th>Purpose</th>
<th>Primer sequences (5'→3')</th>
<th>Annealing temperature (°C)</th>
<th>Product size (bp)</th>
</tr>
</thead>
</table>
| RARβ-M       | Methylation specific | F: TCGAGAGCAGGAGGCGATTCG  
R: GACCCAATCCACCCGAGAACCA  | 60                         | 146                  |
| RARβ-U       | Unmethylation specific | F: TTAGAAATGGTATGTGGATTTG  
R: AAGGAAATCCCTAAAGAAACCA | 60                         | 146                  |
| RARβ         | Bisulfite-modified DNA specific | F: TTAAGGTTCAGGTGGTGAC  
R: GCCAGGTTCTCTAGAGGCTGGTG | 58                         | 256                  |
| GAPDH        | RT-PCR   | F: TTGCAACTGTTTGTATGACTT  
R: AGCAGTGGAAATGTCAAGG | 60                         | 983                  |

*MSP.* MSP takes advantage of the differences between methylated and unmethylated sequences after bisulfite modification (36, 40). To verify the specificity of the PCR reaction, we used placenta DNA as a negative control and 5S-rRNA methylated placenta DNA as a positive control for hypermethylation. A first round of PCR was performed using primers specific for bisulfite-modified DNA (Ref. 32; Table 1). PCR was performed under the conditions as described by Bovenzi and Momparler (32). All reactions contained 5–10 ng of bisulfite-modified DNA and 1.2 units of Qiagen Hotstart Taq polymerase in a 25-μl reaction volume. The DNA fragments resulting from the first PCR were used for MSP. Methylation- and unmethylation-specific primer pairs for the RARβ promoter region (RARβ-M and RARβ-U) were the same as described by Cote et al. (29). These primers were used to amplify a 146-bp fragment of the RARβ promoter region. PCR products were analyzed on a 3% agarose gel with ethidium bromide staining.

Samples with only the methylation-specific band were designated as M, possibly reflecting homozygous hypermethylation; samples with only the unmethylation-specific band were designated as U. If the sample had both methylation- and unmethylation-specific bands, a densitometry visual comparison system was used to determine the band intensities, and the ratio of the methylation-specific and unmethylation-specific band intensities was calculated. We use the designations M/U to indicate that the intensity ratio of M/U is >2, and U/M to indicate that the intensity ratio of U/M is >2.

In Situ Hybridization. The presence of RARβ mRNA in human ESCC samples was analyzed by use of nonradioactive in situ hybridization with a digoxigenin-labeled RARβ cRNA probe as described previously (41, 42). The sense and antisense probes were prepared, and the quality and specificity of the probes were determined by Northern blotting as described previously (43). Either sense probe or probe vehicle was used as a negative control to verify the specificity of the antisense cRNA probe. The stained sections were reviewed under a Nikon microscope. For each case, two pathologists (G-Y. Y. and J. L.) examined the immunohistochemical staining intensity was graded as −, −/+, +, or ++.
Studies with ESCC Cell Lines. The ESCC cell lines KYSE 510, 150, and 450 were maintained in RPMI 1640 and Ham F12 mixed (1:1) medium containing 5% fetal bovine serum. KYSE 150 and 450 cells were harvested on day 3 for total RNA and genomic DNA extraction. For treatment of KYSE 510 cell with DAC, after seeding, medium containing DAC (8.7 μM) was added to the cells on days 1, 3, and 5. Cells were harvested on day 7 for extraction of total RNA and genomic DNA. RARβ mRNA was analyzed with RARβ-specific primers, using the Advantage RT for PCR kit and Advantage PCR kit (Clontech, Palo Alto, CA; Table 1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene, was used as an internal control. PCR products were analyzed on a 2% agarose gel. In these cell samples, methylation status was also determined by MSP assay as described above.

Classification and Statistical Analysis. For methylation status classification, we considered samples with M, M/U, and MU as methylation positive, and those with U/M and U as methylation negative. For in situ hybridization, intense purple staining (+ and + +) was classified as positive; no staining (−) and background or trace faint staining (−/+) were classified as negative. Fisher’s exact test was used to analyze the correlation between DNA methylation and lost expression of the RARβ gene. Differences with \( P < 0.05 \) were regarded as significant.

RESULTS

Hypermethylation of 5′ CpG Islands in the RARβ Gene Locus. The methylation status of the RARβ CpG islands was investigated by MSP in ESCC samples together with their adjacent normal epithelial. Examples of esophageal specimens producing methylation-specific bands, unmethylation-specific bands, or both bands are shown in Fig. 1. The CpG islands in the promoter region of the RARβ gene were methylated in 14 of 20 ESCC samples (70%). Of these 14 samples, 9 showed only the methylation-specific bands (M), and 4 (samples 20, 26, 32, and 40) showed both methylation- and unmethylation-specific bands with similar intensities (MU). One (sample 13) showed a methylation-specific band and a weak unmethylation-specific band (M/U). In the 17 adjacent nontumorous epithelial tissue samples, 14 showed only the unmethylation-specific bands (U), 1 showed a stronger unmethylation-specific band and a weaker methylation-specific band (U/M; sample 22), and 2 showed only the methylation-specific bands (samples 28 and 30).

To further analyze the methylation status, we analyzed laser-capture-microdissected cells from morphologically normal regions of the esophageal epithelium. Of the five normal samples analyzed, three (samples 17, 21, and 26) had only unmethylation-specific bands, one (sample 22) was U/M, and one (sample 30) had only a methylation-specific band (Fig. 1 and Table 3). Microdissected samples with precancerous lesions were also used for the methylation analysis. Of the 21 BCH samples, CpG islands were hypermethylated in 9 (43%), of which sample 22 showed M/U status. Of the 12 DYS samples, 7 (58%) were hypermethylated, of which 3 showed both methylation- and unmethylation-specific bands: sample 26 was classified as M/U, and samples 13 and 22 as M/U.

Expression of RARβ mRNA in Human ESCC Tissues and Precancerous Lesions. We determined RARβ mRNA expression in human esophageal frozen sections containing different lesions, using in situ hybridization. RARβ was expressed in the cytoplasm and around the cell nucleus, and positive cells were distributed in the entire epithelium layer (Fig. 2). RARβ mRNA was expressed in 76% (13 of 17) of normal epithelium samples, 81% (17 of 21) of BCH samples, 50% (4 of 8) of DYS samples, and 42% (10 of 24) of ESCC samples (Table 2). In BCH, the positive staining intensity was generally lower than in normal epithelium (Table 2). The results indicated that in patients with ESCC, RARβ expression was lost in ~20% of samples containing normal epithelium or BCH, but in ~50% of the samples with DYS and ESCC.

Correlation between Hypermethylation and Silencing of RARβ Gene in Esophageal Carcinogenesis. To elucidate the relationship between DNA hypermethylation and loss of expression of RARβ, we compared the results from 13 normal epithelium samples, 6 BCH samples, 7 DYS samples, and 18 ESCC samples (Table 3). The five samples having both methylation- and unmethylation-specific bands with MU status (Table 3) were the most ambiguous ones, with two samples showing −, two showing −/+ and one sample showing + status in in situ hybridization. The appearance of the methylation-specific band was associated with low or no expression of mRNA. It is not clear whether MU status represents heterozygous DNA hypermethylation. With the other 39 samples (Table 3), we observed good correlation between hypermethylation and loss of RARβ mRNA expression in 37 samples. The two exceptions included one “normal” sample (sample 21), which had no hy-
Hypermethylation of RARβ in ESCC

Expression of RARβ in normal esophageal epithelium, DYS, and ESCC as determined with in situ hybridization. Digoxigenin-labeled RARβ cRNA probe was hybridized with human esophageal frozen sections. A and B, normal esophageal epithelium (×200), inset (G; ×400); C and D, DYS (×200); E and F, ESCC (×200). A, C, and E were stained with H&E. B, D, and F show results of in situ hybridization.

DNA hypermethylation (Fig. 3), and KYSE 150 showed both methylation- and unmethylation-specific bands, suggesting that it harbored partial hypermethylation or heterozygosity (Fig. 3) at the CpG islands of the RARβ gene. Reverse transcription-PCR analysis showed that KYSE 510 cells did not have detectable RARβ mRNA expression (Fig. 3), whereas KYSE 150 showed weaker mRNA expression (Fig. 3). The results suggest that heterozygous hypermethylation did not inactivate but lowered the expression of RARβ. To determine whether hypermethylation at the CpG islands of the RARβ promoter region is responsible for silencing the RARβ gene, we treated the cells with DAC, a DNA methyltransferase inhibitor, whereas other cells were resistant to the treatment (45).

### DISCUSSION

Expression of RARβ is critical for retinoic acid-induced cell growth and differentiation. In many cancers, including ESCC, RARβ expression is frequently lost (15). In esophageal carcinogenesis, it is not clear when and how expression of RARβ is altered. In this study, we hypothesize that CpG island hypermethylation of the RARβ gene is a key mechanism for the silencing of this gene, and this process was investigated systematically in human esophageal tissues with different grades of lesions as well as in ESCC cell lines. Hypermethylation of the RARβ promoter region was observed in some of the histologically normal esophageal epithelial samples. In some of these samples, both methylation- and unmethylation-specific bands were observed. An interesting possibility is that hypermethylation had already occurred in some apparently normal cells. The percentage of samples with hypermethylation increased in BCH (43%) and further increased in DYS (58%), which was similar to that in ESCC (70%) samples. Some of the ESCC samples also had both methylation- and unmethylation-specific bands. If we assume that the tumor developed from the same clone of epithelial cells, the minor unmethylation-specific bands (case 13 in

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**Table 2** RARβ expression in esophageal samples with different lesions

<table>
<thead>
<tr>
<th>Sample</th>
<th>−a</th>
<th>−/+</th>
<th>+</th>
<th>++</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (n = 17), n (%)</td>
<td>4 (23.5)</td>
<td>0</td>
<td>0</td>
<td>13 (76.5)</td>
</tr>
<tr>
<td>BCH (n = 21), n (%)</td>
<td>4 (19.0)</td>
<td>0</td>
<td>10 (47.6)</td>
<td>7 (33.3)</td>
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<tr>
<td>DYS (n = 8), n (%)</td>
<td>4 (50.0)</td>
<td>0</td>
<td>4 (50.0)</td>
<td>0</td>
</tr>
<tr>
<td>ESCC (n = 24), n (%)</td>
<td>12 (50.0)</td>
<td>2 (8.3)</td>
<td>6 (26.2)</td>
<td>4 (16.6)</td>
</tr>
</tbody>
</table>

* a−, no staining; −/+ , background or faint staining; +, positive staining; ++ , intense staining.

Samples with + or ++ are considered positive for mRNA expression.
sected samples is technically very difficult and was not success-
cells in the sample. RNA analysis using laser-capture-microdis-
ysis always involves the risk of possible contamination by other
expression in human ESCC samples because PCR-based anal-

Table 3  RARβ gene methylation status and corresponding mRNA expression in sample with different lesions

<table>
<thead>
<tr>
<th>Case no.</th>
<th>MS&quot;</th>
<th>ISH&quot;</th>
<th>Case no.</th>
<th>MS</th>
<th>ISH</th>
<th>Case no.</th>
<th>MS</th>
<th>ISH</th>
<th>Case no.</th>
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<th>ISH</th>
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<tr>
<td>6</td>
<td>U</td>
<td>++</td>
<td>10</td>
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<td></td>
<td>16</td>
<td>M</td>
<td></td>
<td>22</td>
<td>M</td>
<td></td>
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<tr>
<td>10</td>
<td>M</td>
<td></td>
<td>12</td>
<td>U</td>
<td>++</td>
<td>17</td>
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<td>12</td>
<td>U</td>
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<td>13</td>
<td>U</td>
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<td>26</td>
<td>M</td>
<td>++</td>
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<td>+</td>
<td>19</td>
<td>M</td>
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<td>20</td>
<td>U</td>
<td></td>
<td>27</td>
<td>ND</td>
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<td>U</td>
<td>ND</td>
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<td>U</td>
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<td>U/M</td>
<td>+</td>
<td>24</td>
<td>U</td>
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<td>21</td>
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<td>22</td>
<td>M/U</td>
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<td>31</td>
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a MS, methylation status. U, sample with only the unmethylation-specific band; M, sample with only the methylation-specific band. MU indicates the presence of both these bands with intensity ratio <2. M/U indicates that the intensity ratio of M/U is >2; U/M indicates that the intensity ratio of U/M is >2.
b ISH, in situ hybridization. The staining was graded as ++, +, −/+ , and −. c Samples obtained from laser-capture microdissection.
d ND, not determined.

Table 4  Correlation between RARβ gene methylation status and the loss of mRNA expression by Fisher’s exact test

<table>
<thead>
<tr>
<th>Methylation status&quot;</th>
<th>Normal</th>
<th>Precancerous lesions (BCH and DYS)</th>
<th>ESCC</th>
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<tbody>
<tr>
<td></td>
<td>P</td>
<td>N</td>
<td>P</td>
</tr>
<tr>
<td>+</td>
<td>0</td>
<td>2</td>
<td>0</td>
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<td>−</td>
<td>10</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>P</td>
<td>0.0385</td>
<td>0.0008</td>
<td>0.0025</td>
</tr>
</tbody>
</table>

a The methylation status is considered + for M, M/U, and MU; and − for U and U/M as defined in the “Materials and Methods” section. mRNA expression was determined by in situ hybridization. It is considered positive (P) for ++ and +, and negative (N) for − and −/−.

Table 3) are likely attributable to contamination by stromal cells. Another possibility is that in some ESCC samples, only one allele of the RARβ gene is hypermethylated, as we observed in the KYSE 150 cell line (Fig. 3). In this case, the methylation- and unmethylation-specific bands are expected to be of equal intensity. This was observed in cases 20, 26, 32, and 40 (Table 3).

We used in situ hybridization to determine RARβ mRNA expression in human ESCC samples because PCR-based analysis always involves the risk of possible contamination by other cells in the sample. RNA analysis using laser-capture-microdissected samples is technically very difficult and was not success-

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ful in the preliminary experiment. Loss of expression of RARβ was observed in samples with histologically normal epithelium and BCH (19 and 24%, respectively) and at increased frequencies in DYS and ESCC samples (43 and 58%, respectively). Our results for samples with lesions were similar to those reported by Qiu et al. (15). The loss of RARβ expression in normal esophageal epithelium has not been reported previously.

The loss of RARβ mRNA expression correlated well with DNA hypermethylation in Fisher’s exact test, showing statistical significance for samples in each category of normal, precancerous lesions, or ESCC. A more rigorous correlation analysis is planned to determine whether hypermethylation is correlated with the loss of RARβ expression in each individual sample. The results from the five samples bearing MU status suggest that methylation of the RARβ promoter decreases the level of mRNA expression. Of the 39 samples with clear methylated or
unmethylated status, 37 showed a good concordance with the mRNA level. In two samples that did not show a concordance, one normal sample had no mRNA expression but showed no hypermethylation. This could be attributable to other mechanisms, such as gene deletion or mutation. The other, an ESCC sample with strong methylation bands but also with RARβ mRNA expression, is more intriguing. One possibility is that the samples were taken from different parts of a heterogeneous carcinoma.

All of the above results suggest that hypermethylation of the RARβ promoter occurs rather early in some individuals and that the frequency of this event increases during esophageal carcinogenesis. The mechanism for this hypermethylation reaction is not known. Esophagitis is rather common in this high-risk population in Linzhou (46, 47), and chronic inflammation could be a driving force for the hypermethylation. It has been suggested that increased cell turnover induced by chronic inflammation could make some CpG islands progressively lose protection against methylation (48).

The involvement of CpG island hypermethylation in the silencing of the RARβ gene is also supported by the observations that treatment of KYSE 510 cells (with hypermethylation of the RARβ promoter and no mRNA expression) with DAC caused partial reversion of RARβ promoter hypermethylation and reexpression of mRNA. Silencing of the RARβ gene would affect the normal differentiation of esophageal epithelial cells and promote carcinogenesis. If hypermethylation-associated silencing of the RARβ gene is a significant event in esophageal carcinogenesis, we may expect that once such an event occurred in an individual’s esophageal epithelium, it should also be observed in samples with more severe lesions. This was indeed observed in most of the esophageal samples (Table 3), but there were some exceptions. In two cases (19 and 30), hypermethylation was observed in normal and DYS samples, but not in the corresponding ESCC samples. It is possible that the group of cells with hypermethylation in normal or DYS tissues, in the absence of other key molecular alterations, did not progress or had not progressed to cancer and that the clinically observed carcinomas originated from different clones of epithelial cells. The reversion of hypermethylation may also occur in the normal sample, but this is less likely in the DYS sample.

Our results suggest that DNA hypermethylation of RARβ promoter CpG islands, which leads to gene silencing, is a rather early event in esophageal squamous carcinogenesis. Dietary or pharmaceutical agents that can inhibit this process may prevent or delay the development of ESCC. In cancer patients, activation of RARβ expression has been proposed as a therapeutic approach (49, 50). Our results provide support for the use of a selected DNA methyltransferase inhibitor for the prevention and treatment of esophageal cancer.

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We thank Dr. Yutaka Shimada (Kyoto University, Kyoto, Japan) for providing the KYSE cancer cell lines for this study.

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