Hypermethylation of the \textit{Retinoic Acid Receptor-\(\beta_2\)} Gene in Head and Neck Carcinogenesis

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\textbf{ABSTRACT}

Purpose: Retinoic acid receptor-\(\beta_2\) (RAR-\(\beta_2\)) expression is suppressed in oral premalignant lesions and head and neck squamous cell carcinomas (HNSCCs). This study was conducted to determine whether RAR-\(\beta_2\) gene expression in such lesions can be silenced by promoter methylation.

Experimental Design: RAR-\(\beta_2\) methylation was analyzed in DNA samples from 22 pairs of primary HNSCC and adjacent normal epithelium, 124 samples of oral leukoplakia, and 18 HNSCC cell lines using methylation-specific PCR. RAR-\(\beta_2\) promoter was methylated in 67, 56, and 53\% of HNSCC tumors, HNSCC cell lines, and microdissected oral leukoplakia specimens, respectively. RAR-\(\beta_2\) hypermethylation was confirmed by sodium bisulfite-PCR combined with restriction enzyme digestion analysis and by random cloning and sequencing of bisulfite-treated DNA isolates.

Results: Significantly higher RAR-\(\beta_2\) hypermethylation levels were found in tumor tissue compared with adjacent normal tissue (\(P = 0.002\)). RAR-\(\beta_2\) methylation in the cell lines was correlated with loss of RAR-\(\beta_2\) expression (\(P = 0.013\)) and inversely related to the presence of mutated p53 (\(P = 0.025\)). The demethylating agent 5-aza-2'-deoxycytidine (5-aza-CdR) restored RAR-\(\beta_2\) inducibility by all-trans-retinoic acid (ATRA) in some of the cell lines, which possess a methylated RAR-\(\beta_2\) promoter. In some cell lines, this effect was associated with increased growth inhibition after combined treatment with 5-aza-CdR and ATRA.

Conclusions: RAR-\(\beta_2\) silencing by methylation is an early event in head and neck carcinogenesis; 5-Aza-CdR can restore RAR-\(\beta_2\) inducibility by ATRA in most cell lines, and the combination of 5-aza-CdR and ATRA is more effective in growth inhibition than single agents.

INTRODUCTION

Retinoids, vitamin A metabolites, and synthetic analogues suppress carcinogenesis in a variety of epithelial tissues, including the oral mucosa (1). Retinoids also inhibit the growth of human head and neck squamous cell carcinoma (HNSCC) cells in nude mice (2). Furthermore, clinical studies have shown that retinoids suppress oral premalignant lesions (e.g., leukoplakia) and decrease the incidence of second primary tumors in patients who had been treated earlier for primary head and neck cancers (3, 4). These effects of retinoids are the likely consequence of their ability to modulate cell proliferation, differentiation, and apoptosis (5).

Retinoids suppress the growth and the squamous differentiation of HNSCC cells \textit{in vitro} (6) and \textit{in vivo} (7). It is thought that many of the effects of retinoids are mediated by nuclear receptors, which include three retinoic acid receptors (RARs), designated as RAR-\(\alpha\), RAR-\(\beta\), and RAR-\(\gamma\), and three retinoid X receptors (RXRs), designated as RXR-\(\alpha\), RXR-\(\beta\), and RXR-\(\gamma\) (5, 8, 9). These receptors form RXR-RAR heterodimers, which bind to specific DNA sequences called RAR elements (5, 8, 9). Transcription regulation by retinoid receptors is determined by interplay of cofactors with opposite effects. In the absence of ligand, corepressors bind to complexes formed between retinoid receptors and response element and suppress transcriptional activation. However, ligand binding causes corepressors to dissociate and coactivators to associate with the retinoid receptors and activate the transcriptional machinery (5, 8, 9).

Abnormalities in the expression or function of retinoid receptors, especially the suppression of the expression of RAR-\(\beta\), have been found in various cancer cell types (10), including premalignant oral lesions (11–15) and HNSCC cell lines (11, 16, 17) and \textit{in vivo} lesions (12). It has been suggested that this change may lead to resistance to some effects of retinoids (11, 12). The loss of RAR-\(\beta\) expression in oral premalignant cells in short-term \textit{in vitro} culture has been associated with immortalization (13, 14). Retinoic acid added at pharmacological doses was able to induce RAR-\(\beta\) expression in some HNSCC cell lines (11, 16) and in premalignant lesions \textit{in vivo} (12). This induction has been shown to be important for the growth inhibitory effects of retinoic acid (18). Furthermore, transfection of RAR-\(\beta\) into various cancer cell types (19–22), including HNSCC cell lines (23, 24), restored responsiveness to all-trans-retinoic acid (ATRA) as evidenced by suppressed cell
proliferation, modulated squamous differentiation, enhanced apoptosis, and suppressed tumorigenicity. On the basis of the above findings, it has been suggested that RAR-β can act as a tumor suppressor.

RAR-β is expressed in several isoforms designated RAR-β1, RAR-β2, and RAR-β3 (5, 8, 9). These isoforms are generated by the usage of different promoters or by alternative splicing. The P2 promoter from which RAR-β2 and RAR-β3 are transcribed includes a DR5 RAR element, and the transcription of these isoforms is enhanced by the binding of RXR-RAR heterodimer to DR5 in the presence of ATRA (25). Indeed, low levels of ATRA resulted in a decreased RAR-β expression in vitro (26) and in vivo (27) and retinoic acid supplementation was able to overcome this suppression by inducing RAR-β in certain HNSCC cell lines in vitro (26) and in premalignant oral cells in vivo (12). However, there are cells, including lung and head and neck carcinoma cell lines, in which even pharmacological ATRA concentrations cannot induce RAR-β (11, 28, 29), suggesting that the suppression of RAR-β expression is mediated by mechanisms other than low ATRA levels.

Recently, an epigenetic mechanism has been identified for the inactivation of tumor suppressor genes. This mechanism is based on the enzymatic addition of methyl groups to deoxy- cytidine residues in the dinucleotide CpG. The aberrant methylation of all or even only a few of the CpG islands may result in a closed chromatin structure and consequently in silencing the transcription of all or even only a few of the CpG islands (30–32). Aberrant hypermethylation of CpG islands has been found in several tumor suppressor genes. This hypermethylation correlates with transcriptional repression that can serve as an alternative to coding region mutations for inactivation of tumor suppressor genes (30–32).

The demethylating agent 5-aza-2'-deoxycytidine (5-aza-CdR) can reactivate the transcription of genes silenced by methylation and lead to restoration of tumor suppressor functions (33). Furthermore, demethylating agents have a potential therapeutic use (33–35).

Several recent studies have demonstrated that the RAR-β gene promoter is hypermethylated in colon, breast, and lung cancers (36–44) and that this methylation could account at least in part for the suppression of RAR-β expression in these cells.

In the present study, we investigated the role of hypermethylation of CpG islands in head and neck carcinogenesis and its association with RAR-β2 expression. In addition, we examined whether the repression of RAR-β2 transcription could be reversed by 5-aza-CdR alone or combined with ATRA, and finally, we evaluated the effects of ATRA and 5-aza-CdR alone and in combination on the growth of HNSCC cell lines.

MATERIALS AND METHODS

**HNSCC Cell Lines and Culture Conditions.** The University of Michigan squamous cell carcinoma (UMSCC) cell lines: UMSCC10A; UMSCC10B; UMSCC11B; UMSCC14B; UMSCC17A; UMSCC17B; UMSCC19; UMSCC22A; UMSCC22B; UMSCC30; UMSCC35, and UMSCC38 were kindly provided by Dr. Thomas Carey (University of Michigan, Ann Arbor, MI). Details about these cells, including karyotyping, were described previously (45–47). The numbers in the cell line designations represent individual patients, and the suffixes “A” and “B” indicate that the cell lines were derived from the original primary tumor and from a lymph node metastasis, respectively. Cell lines 183A, MDA886Ln, 1186, and 1483 were kindly provided by Dr. Peter Sachs (New York University College of Dentistry, New York, NY; Refs. 48, 49). Cell line TR146 was kindly provided by Dr. Alfonse Balm (Free University of Amsterdam, Amsterdam, the Netherlands; Ref. 50), and cell line SqCC/Y1 was kindly provided by Dr. Michael Reiss (Yale University, New Haven, CT; Ref. 51). The cells were grown in a monolayer culture in a 1:1 mixture (vol/vol) of DMEM and Ham’s F12 medium containing 5% fetal bovine serum and antibiotics at 37°C in a humidified atmosphere composed of 95% air and 5% CO₂.

**ATRA and 5-Aza-CdR Treatment for RAR-β Induction.** ATRA obtained from Dr. Werner Bollag (F. Hoffman-La Roche, Basel, Switzerland) was dissolved in DMSO at a concentration of 10 mM and stored under N₂ in the dark at −80°C. The stocks solution was diluted to 1 µM with growth medium just before use. 5-Aza-CdR (Sigma, St. Louis, MO) was dissolved in cold RPMI 1640 immediately before use. HNSCC cells were grown in a medium containing 0.5 µM 5-aza-CdR for 4 days with one refeeding of fresh medium on day 3 before genomic DNA, and total RNA was extracted and tested for methylation status as well as restoration of RAR-β expression [reverse transcription-PCR (RT-PCR) and Northern blotting]. In some experiments, cells were treated first with 0.5 µM 5-aza-CdR for 3 days and then with 0.5 µM 5-aza-CdR plus 1 µM ATRA for 1 day.

**DNA Extraction from Cell Lines and Frozen and Paraffin-Embedded Tissue Samples.** Genomic DNA was extracted and purified from 18 HNSCC cell lines using a Stratagene DNA extraction kit according to the manufacturer’s instructions (Stratagene, La Jolla, CA). For the isolation of genomic DNA, we used 22 matched pairs of frozen tumor specimens and their adjacent normal mucosa tissue obtained as a separate biopsy from the same patients taken outside of the margin. These samples were examined histologically and found to appear normal. The frozen samples were not microdissected. Five paraffin-embedded primary HNSCC samples lacking the adjacent normal tissue were also analyzed. All tissue samples were ground and incubated for 3 h in a lysis buffer containing 1% SDS, 0.1 mM NaCl, 50 mM EDTA (pH 8.0), and 200 µg/ml proteinase K. The DNA was then purified with phenol/chloroform using standard procedures and precipitated with ethanol. DNA was extracted from the paraffin-embedded samples using the Intergen EX-WAX DNA extraction kit (Intergen Co., Purchase, NY) according to the manufacturer’s instructions.

**Oral Leukoplakia Tissue Microdissection and DNA Extraction.** Biopsies were obtained at baseline from 124 patients who participated in a prospective oral premalignancy chemoprevention trial in which patients were treated with isotretinoin (13-cis-retinoic acid) or β carotene plus retinyl palmitate between the years 1992 and 1999. Clinical responses were evaluated after 3 months. The major characteristics of this population are presented in Table 2. The biopsies had been fixed in formalin and embedded in paraffin. Thin sections (4-µm thick) were prepared from the paraffin blocks and stained with H&E. The epithelial part of each biopsy section was microdissected by using a 251/2 gauge steel needle under a stereo-
microdissected and disrupted in 100 μl of a lysis buffer containing 50 mM Tris-HCl (pH 8.0), 1% SDS, and 0.5 mg/ml proteinase K and incubated at 42°C for 24 h. The digested products were purified by extraction with phenol:chloroform twice. DNA was then precipitated by the ethanol precipitation method in the presence of glycogen (Roche Molecular Biochemicals, Indianapolis, IN) and recovered in distilled water.

Methylation-Specific PCR (MSP) and Bisulfite-PCR for Restriction Analysis. The MSP assay takes advantage of DNA sequence differences between methylated and unmethylated alleles after bisulfite modification, which can change the cytosine base to uracil base if the original DNA was unmethylated, whereas this change does not occur if the original DNA is methylated. The methylation status of RAR-β was determined by the MSP as described by Herman et al. (52). In brief, after genomic DNA isolation and purification, 2 μg of genomic DNA were treated with sodium bisulfite for 16 h. After purification, a 2-μl aliquot was used as a template for PCR using primers to differentiate between methylated and unmethylated extracted DNA for RAR-β 5ꞌ-promoter region as described by Cote et al. (37). The modified primers for methylated sodium bisulfite DNA that provided 146 bp of the transcript were the following: 5ꞌ-TCGAGAACGCGACGTTCGC-3’ (sense) in position 950–969 and 5ꞌ-GACCAATCCAACCGAAGCA-3’ (antisense) in position 1095–1076, whereas those for unmethylated sodium bisulfite DNA were 5ꞌ-TTGAGAATGTAGTGATT-TGA-3’ (sense) in position 950–970 and 5ꞌ-ACCAATC-CAACCAAAACA-3’ in position 1095–1076. The annealing temperatures for methylated and unmethylated modified DNA were 58°C and 50°C, respectively. In addition, methylation of RAR-β2 promoter region was determined using bisulfite-PCR followed by restriction digestion as described previously (37, 52, 53). In brief, 20–40 μl of the amplified products were digested with the restriction enzyme TarI (MaeII), (Fermentas, Inc., Hanover, MD) which distinguishes methylated sequences from unmethylated ones, using the primers 5ꞌ-CCAAATTCTCCTTACATCCC-3’ (sense) and 5ꞌ-CCAAATTCCTCTTCCAAAATAA-3’ (antisense), which yield a 207-bp product of sodium bisulfite modified DNA. The PCR products were electrophoresed on a 3% agarose or 5% acrylamide gel and visualized using ethidium bromide staining. Lymphocyte DNA, methylated in vitro by CpG methylase (SssI; New England Biolabs, Inc., Beverly, MA) was used as a positive control. A negative control (without DNA) was included in every set of PCR experiment. Other details of the experimental protocols are described on our web site.

DNA Cloning and Sequencing. The above-mentioned primers 5ꞌ-AAGTATAGGAGTGGGTGGTTA-3’ (sense) and 5ꞌ-CCAAATTCCTCTTCCAAAATAA-3’ (antisense) were used to clone the bisulfite-modified 207 bp of DNA. The method has the advantage of being able to analyze the maximum number of CpG islands in terms of methylation. PCR fragments (200 ng) were cloned into a pCR2.1 TOPO vector (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Plasmid DNA was extracted and purified using the Quiagen Plasmid Mini Kit (Qiagen, Valencia, CA), and the sequence of 20–30 clones was determined for every PCR product using the ABI Prism 377 DNA sequencer (Applied Biosystems, Foster, CA) to determine the percent and site of methylated CpG islands.

RNA Purification, RT-PCR, and Northern Blot Analysis. Total RNA from different cell lines was isolated using the Tri Reagent solution (Molecular Research Center, Inc., Cincinnati, OH) and tested for integrity and purity before being subjected to a RT-PCR reaction. The latter was performed as per manufacture’s instruction using access RT-PCR system (Promega, Madison, WI) at an annealing temperature of 68°C for 30 cycles. The sense oligonucleotide used in the detection of the 195-nucleotide fragment originating from the human RAR-β2 cDNA was 5ꞌ-CAAACGGAATGCGACCATC-3’, which corresponded to position 592–612 in exon 4 of RAR-β2 as numbered by Naggal et al. (54), the antisense oligonucleotide used in the detection of RAR-β2 was 5ꞌ-CGGCAGACGCAGTGAATCTCA-3’ and 5ꞌ-CAGGTTCCAGGTTCTGTC-3’, corresponding to positions 189–210 and 331–311, respectively, as numbered by Chen et al. (55) were used for the detection of the 143-nucleotide fragment originating from the human S14 ribosomal protein cDNA as an internal control.

Each sample included reverse transcription-negative controls in which the reverse transcriptase was omitted from the initial reverse transcription step.

Northern blot analysis was performed by subjecting 20 μg of total RNA to electrophoresis in 0.66 M formaldehyde on a 1.2% agarose gel. The RNA was then transferred to a membrane (Maximum Strength Nytran Plus; Schleicher & Schuell, Keene, NH) and UV cross-linked and hybridized with a probe for the RAR-β and glyceraldehyde-3-phosphate dehydrogenase, as an internal control, labeled with 32PdCTP (ICN Biomedicals, Costa Mesa, CA) as detailed previously (56).

Growth Inhibition Assay. Cells were seeded at densities ranging from 5 × 104 to 10 × 104 cells/well in 96-well tissue culture plates. After 24 h, the cells were randomly divided into five groups (A–E) in triplicate wells and treated for 3 days in medium containing 5% fetal bovine serum and supplemented as follows: group A and B, DMSO as solvent control; C, 1 μM ATRA; and D and E, 0.5 μM 5-aza-CdR. After 3 days, the medium was replaced with fresh medium containing the following additives: group A received DMSO as control; B and C, 1 μM ATRA; D, 0.5 μM 5-aza-CdR; and E, 0.5 μM 5-aza-CdR plus 1 μM ATRA. After an additional 4-day incubation, cell numbers were estimated using the sulforhodamine B assay as described in detail previously (55). The percentage of growth inhibition was calculated using the following equation: percentage of growth inhibition = (1-At/Ac) × 100 in which Ac and At represented the absorbance in treated and control culture, respectively.

Detection of p53 Point Mutations. p53 mutations were detected using PCR amplification and sequencing of genomic DNA using a primer set that can determine deletion of p53 from exon 4 to exon 9. The sense primer used was 5ꞌ-
TTCACCTTGCCCTGACTT-3′ and the antisense primer was 5′-CTGGAAACTTTCCACTTGAT-3′. The PCR conditions and sequencing techniques used were described in detail previously (57).

**Statistical Analysis.** Statistical analysis was performed using Fisher’s exact test to examine the association between two categorical variables. McNemar’s test was applied for testing the change over time of a categorical variable. Wilcoxon rank-sum test was used for comparing the distribution of a continuous variable between two groups. All analyses were done using the SAS software (SAS Institute, Inc., Cary, NC). Two-sided tests were used to calculate the P, and P < 0.05 was considered statistically significant.

**RESULTS**

**RAR-β Promoter Methylation in HNSCC Tumor Samples and Adjacent Normal Mucosa.** The methylation status of CpG sites in the RAR-β2 promoter in 27 primary head and neck tumors and 22 adjacent normal tissues was determined using the MSP method. Selected examples of analysis of 5 cases are presented in Fig. 1. The detection of a 146-bp fragment (Fig. 1, top panel) indicates that the DNA was methylated in all five tumor samples and in two of the five adjacent normal specimens. Although unmethylated DNA was detected in all specimens, the tumor cells appeared to express less of this type of PCR product than the normal samples. The data on the analysis of samples from the 27 cases are shown in Table 1. Methylation of RAR-β2 was observed in 18 of the 27 tumor samples (66%). Among the 22 paired cases, 10 (45%) showed methylation in tumor but not in adjacent normal tissues. Methylation was detected in normal tissue in 6 of 22 cases (27%). In all of the cases in which normal tissue was methylated, tumor tissue was also methylated. The remaining 6 cases had no RAR-β2 methylation in both normal and tumor tissues (Table 1). McNemar’s test showed that RAR-β2 methylation is significantly higher in tumor tissue than in normal tissue (P = 0.002). Methylation of RAR-β2 was detected in tumors that had developed in different anatomical sites in the head and neck region.

**RAR-β Promoter Methylation in Oral Leukoplakia Specimens.** The methylation status of RAR-β2 promoter was analyzed by MSP in samples derived from microdissected epithelial cells from oral leukoplakia specimens (Fig. 2). Sixty-six of 124 specimens (53%) showed methylation of RAR-β2. There was no statistically significant correlation between methylation of RAR-β2 and age, sex, race, histological type, site, tobacco use, or alcohol consumption (Table 2). A slightly higher proportion of methylation was found in dysplasia samples versus hyperplasia samples (60% versus 50%), but the difference was not statistically significant. The vast majority of dysplasia samples (80%) were mild dysplasia.

The 3-month clinical response rate was higher in the RAR-β2-methylated group than the unmethylated group (52% versus...
To study the methylation status of HNSCC cell lines, we prepared bisulfite DNA and used it for PCR amplification followed by restriction enzyme analysis of the PCR product using the TstI restriction enzyme, which digests DNA at the 5′-ACGT-3′ site. The promoter region, CpG sites, primers used for cloning, PCR product, and restriction enzyme site are shown in Fig. 3A. Fig. 3B shows the methylation pattern of selected cell lines. The cell lines examined showed variation in the degree of promoter methylation as indicated by the relative intensity of bands representing PCR products of methylated and unmethylated CpG sites in the promoter. Ten of 20 (65%) cell lines showed RAR-β2 promoter hypermethylation (Table 3).

Fig. 4 shows the sequencing histogram for 11 CpG sites examined in the 5′-promoter region of RAR-β. Partial methylation (coexistence of methylated and unmethylated alleles) was observed in the MDA866Ln, SqCC/Y1, UMSCC17A, UMSCC 22A, and MDA 1186. High-density promoter methylation (>80% methylated alleles) was observed in UMSCC38, UMSCC 35, UMSCC 17B, UMSCC 22B, and TR146 cell lines. No promoter methylation was observed in UMSCC10A, UMSCC10B, UMSCC11B, UMSCC14B, UMSCC19, UMSCC 30, 183A, and 1483 cell lines (Table 3). In addition, Fig. 4B shows the proportion of clones of PCR products with different degrees of methylation of the 11 CpG sites among the total number of colonies analyzed. The UMSCC38 cells appear to have 73% fully methylated clones and 24% mostly methylated clones. In contrast, the 183A cells have 95% fully unmethylated clones and only 5% partially methylated clones.

**Correlation between p53 Mutation and RAR-β Methylation in HNSCC Cells.** p53 mutation analysis showed that 10 of 18 (56%) of the HNSCC lines had a mutation in the open reading frame of the gene (Table 3). There was a significant correlation between p53 mutation and lack of RAR-β methylation and vice versa [p53 mutation found in 7 of 8 (88%) in the unmethylated cells and only 3 of 10 (30%) in the methylated cells, P = 0.025, Fisher’s exact test]. An analysis of p53 mutations in 10 primary tumors did not show an inverse correlation with RAR-β methylation (data not shown).

**Constitutive and Induced Expression of RAR-β2 in HNSCC Cells.** The constitutive level of RAR-β2 expression was in all of the 18 cell lines was investigated using both RT-PCR and Northern blotting. Examples of the results are presented in

<table>
<thead>
<tr>
<th>Table 2 Patient characteristics and their relationship with retinoic acid receptor-β2 (RAR-β2) methylation in 124 oral leukoplakia patients</th>
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<tbody>
<tr>
<td><strong>RAR-β2 methylation</strong></td>
</tr>
<tr>
<td><strong>Age</strong></td>
</tr>
<tr>
<td><strong>Sex</strong></td>
</tr>
<tr>
<td><strong>Race</strong></td>
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<tr>
<td><strong>Histologic type</strong></td>
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<tr>
<td><strong>Site</strong></td>
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<tr>
<td><strong>Tobacco use</strong></td>
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<td><strong>Alcohol consumption</strong></td>
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</tbody>
</table>

* Fisher’s exact test unless specified otherwise.
* Fisher’s exact test.
* The dysplasia lesions were mostly mild dysplasia (80%).
* One patient did not have tobacco and alcohol information.

31%, P = 0.03, Fisher’s exact test). Similar pattern was found between the isotretinoin arm and the β-carotene plus retinyl palmitate arm. No statistical significance was found between the clinical response and RAR-β methylation in later time points. In addition, no statistically significant difference in time-to-progression was found between the methylated group and unmethylated group in all 124 patients as well as in 82 patients with hyperplasia or in 42 patients with dysplasia.

**RAR-β2 Promoter Methylation in HNSCC Cell Lines.**

To study the methylation status of HNSCC cell lines, we prepared bisulfite DNA and used it for PCR amplification followed by restriction enzyme analysis of the PCR product using the TstI restriction enzyme, which digests DNA at the 5′-ACGT-3′ site. The promoter region, CpG sites, primers used for cloning, PCR product, and restriction enzyme site are shown in Fig. 3A. Fig. 3B shows the methylation pattern of selected cell lines. The cell lines examined showed variation in the degree of promoter methylation as indicated by the relative intensity of bands representing PCR products of methylated and unmethylated CpG sites in the promoter. Ten of 18 (65%) cell lines showed RAR-β2 promoter hypermethylation (Table 3).

![Fig. 3](image-url) A, sequence of part of the promoter-exon region of the RAR-β2 gene (GenBank accession no. X56849), which was subjected to bisulfite treatment. The forward and reverse primers used to clone individual bisulfited DNA are underlined, the modified nucleotides are indicated by a capital T, the methylated cytosines are indicated by a bold capital C, and the Tail restriction enzyme site (CGT) is italicized and underlined. B, methylation analysis of the promoter-exon region part in selected head and neck squamous cell carcinoma cell lines (indicated above each lane). The PCR products from methylated cell lines were digested by Tail restriction enzyme, giving rise to two bands: the upper unmethylated DNA populations and the lower methylated DNA populations. Tail was unable to digest the DNA from unmethylated cell lines giving rise to single discrete bands of 207 bp. The suffix U and M represents the unmethylated and methylated fragments, respectively.
We found that promoter methylation was correlated with RAR-\(\beta_2\) expression silencing [RAR-\(\beta_2\) expression was lost in 2 of 8 (25%) in the unmethylated cells and 9 of 10 (90%) in the methylated cells, \(P = 0.013\), Fisher’s exact test], suggesting that methylation is the major mechanism responsible for suppression of RAR-\(\beta_2\) expression in the HNSCC cell lines. Indeed, RAR-\(\beta_2\) was induced by ATRA 3–10-fold in 22B and 38A HNSCC cell lines after treatment with the demethylating agent 5-aza-CdR (Figs. 5B and 6). HNSCC cell lines without promoter methylation (e.g., 183A and 1483) showed constitutive expression of RAR-\(\beta_2\) and also induction of RAR-\(\beta_2\) by ATRA alone. The induction of RAR-\(\beta_2\) by 1 \(\mu\)M ATRA was not enhanced by...

### Table 3: Relationship between cell homozygosity, retinoic acid receptor-\(\beta_2\) (RAR-\(\beta_2\)) expression, methylation status, and p53 mutations in head and neck squamous cell carcinoma cell lines

<table>
<thead>
<tr>
<th>No.</th>
<th>Cell line</th>
<th>Primary site</th>
<th>No. of 3p24 loci(^a)</th>
<th>RAR-(\beta_2) status(^b)</th>
<th>Methylation status(^c)</th>
<th>p53 mutation(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>UMSCC10A</td>
<td>Larynx</td>
<td>2</td>
<td>–</td>
<td>U</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>UMSCC10B</td>
<td>Larynx</td>
<td>2</td>
<td>+</td>
<td>U</td>
<td>+</td>
</tr>
<tr>
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<td>UMSCC17A</td>
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<td>–</td>
<td>M</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>UMSCC17B</td>
<td>Larynx</td>
<td>1</td>
<td>–</td>
<td>MM</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>MDA886La</td>
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<td>ND(^*)</td>
<td>+</td>
<td>M</td>
<td>–</td>
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</tr>
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<td>–</td>
<td>M</td>
<td>–</td>
</tr>
<tr>
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<td>UMSCC22B</td>
<td>Hypopharynx</td>
<td>1</td>
<td>–</td>
<td>MM</td>
<td>–</td>
</tr>
<tr>
<td>9</td>
<td>UMSCC35</td>
<td>Oropharynx</td>
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<td>–</td>
<td>MM</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
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<td>Floor of mouth</td>
<td>1</td>
<td>+</td>
<td>U</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
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<td>Base of tongue</td>
<td>1</td>
<td>–</td>
<td>U</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>UMSCC30</td>
<td>Pyriform sinus</td>
<td>1</td>
<td>+</td>
<td>U</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>UMSCC38</td>
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<td>–</td>
<td>MM</td>
<td>–</td>
</tr>
<tr>
<td>14</td>
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<td>Tonsil</td>
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<td>+</td>
<td>U</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
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<td>Epiglottis</td>
<td>ND</td>
<td>–</td>
<td>M</td>
<td>+</td>
</tr>
<tr>
<td>16</td>
<td>1483</td>
<td>Retromolar trigone</td>
<td>ND</td>
<td>+</td>
<td>U</td>
<td>–</td>
</tr>
<tr>
<td>17</td>
<td>SqCC/Y1</td>
<td>Buccal mucosa</td>
<td>ND</td>
<td>–</td>
<td>M</td>
<td>+</td>
</tr>
<tr>
<td>18</td>
<td>TR146</td>
<td>Buccal mucosa</td>
<td>ND</td>
<td>–</td>
<td>MM</td>
<td>–</td>
</tr>
</tbody>
</table>

\(^a\) One copy of DNA at the 3p24 locus (29, 45–47).

\(^b\) RAR-\(\beta_2\) expression level as detected using both Northern blotting and reverse transcription-PCR analysis and before treatment.

\(^c\) U, unmethylated; M, partially methylated with a mix of methylated and unmethylated alleles; MM, high-density, >80% methylated alleles.

\(^d\) Absence (−) or presence (+) of p53 mutation (57).

\(^*\) ND, not determined.

Figs. 5A and 6, and the summary of the data are presented in Table 3.

We found that promoter methylation was correlated with RAR-\(\beta_2\) expression silencing [RAR-\(\beta_2\) expression was lost in 2 of 8 (25%) in the unmethylated cells and 9 of 10 (90%) in the methylated cells, \(P = 0.013\), Fisher’s exact test], suggesting that methylation is the major mechanism responsible for suppression of RAR-\(\beta_2\) expression in the HNSCC cell lines. Indeed, RAR-\(\beta_2\) was induced by ATRA 3–10-fold in 22B and 38A HNSCC cell lines after treatment with the demethylating agent 5-aza-CdR (Figs. 5B and 6). HNSCC cell lines without promoter methylation (e.g., 183A and 1483) showed constitutive expression of RAR-\(\beta_2\) and also induction of RAR-\(\beta_2\) by ATRA alone. The induction of RAR-\(\beta_2\) by 1 \(\mu\)M ATRA was not enhanced by...
after 5-aza-CdR treatment. S14 served as an internal control.

the unmethylated 183A cell line did not show any differences before and methylated UMSCC38 cell line after 5-aza-CdR treatment. As expected, with 5-aza-CdR, respectively. Note the expression of the completely

/H9262 treatment at 1

gesting that RAR-

/H9252 (5-aza-CdR) treatment at 1

2 methylation is an

lesions. These results indicate that RAR-

/H9252 expression associated

ADDITIONAL METHODS

To determine whether pretreatment with 5-aza-CdR can sensitize HNSCC cells to ATRA, we analyzed the effects of 5-aza-CdR alone, ATRA alone, and a sequential treatment with 5-aza-CdR and 5-aza-CdR plus ATRA on the growth of seven HNSCC cell lines. We first analyzed the effects of different 5-aza-CdR concentrations and selected 0.5 μM for this experiment because it caused 40–55% growth inhibition in most HNSCC cell lines (Fig. 7). Treatment with 1 μM ATRA for 3 days was only effective in inhibiting the growth of 22A, 22B, and 183A cell lines. However, a 7-day treatment with 1 μM ATRA inhibited the growth of the latter cell lines and in addition also of the 38A cells (Fig. 7). Pretreatment with 5-aza-CdR followed by cotreatment with 5-aza-CdR and ATRA resulted in greater growth inhibition than each agent alone in most cell lines, except the UMSCC17A cells in which RAR- expression was not detectable after treatment with ATRA alone, 5-aza-CdR alone, or sequential/combined 5-aza-CdR/ATRA combination.

DISCUSSION

In this study, we have demonstrated that RAR- is silenced by methylation of CpG islands in its promoter in 66% of HNSCC cell lines. Furthermore, this epigenetic change was detected in >50% of HNSCC tumors and premalignant oral lesions. These results indicate that RAR- methylation is an early event in head and neck carcinogenesis and that malignant transformation only increases the methylation from 53 to 66%. However, because the HNSCC specimens were derived from primary cancers in a variety of sites compared with leukoplakia that were all oral, it is not possible to determine conclusively whether the increase in methylation in tumors versus leukoplakia represents progression or differential location. Nonetheless, it is noteworthy that all seven primary tumors from oral origin had a high promoter methylation, suggesting that methylation increases during oral carcinogenesis.

Our finding that RAR- promoter is methylated in 27% of adjacent, normal-appearing epithelial tissues may reflect very early changes that may even precede the appearance of histologically recognizable premalignant leukoplakias.

The study also demonstrated that the epigenetic silencing of RAR- expression can be reversed by the potent reversible demethylating agent 5-aza-CdR combined with ATRA. In addition, we have demonstrated that a sequential/concurrent treatment of HNSCC cell lines with 5-aza-CdR and ATRA results in greater growth inhibitory effects than each agent alone.

Although tumor suppressor genes may be inactivated by mutation, it is increasingly accepted that those genes may undergo inactivation through an epigenetic mechanism (30–32). Several studies described the finding that the promoter of RAR- gene is methylated in various cancers, and a few of these studies also found that methylation was associated with RAR-
expression silencing (36–44). However, the timing of RAR-β2 silencing relative to the stage of carcinogenesis has received limited attention. Thus, RAR-β2 methylation was detected in 11% of low-grade cervical intraepithelial neoplasias, 29% of high-grade cervical intraepithelial neoplasias, and 26% of invasive cervical cancers (58). It appears that the extent of RAR-β2 methylation in early as well as advanced head and neck lesions detected in our study is much greater than that reported in the cervix (58). A recent study in vitro using explant cultures of early lesions of the oral cavity, mostly leukoplakias with dysplasia, has shown that RAR-β expression was lost in cultures of immortal cells and HNSCC cell lines and could be restored in a few immortal dysplasia cultures after 7 daily treatments with 1 μM 5-aza-CdR. However, RAR-β expression could not be restored by a similar treatment in any of four HNSCC cell lines (14). Our findings with some HNSCC cell lines are at variance with those of McGregor et al. (14) in that we were able to restore RAR-β2 expression in several HNSCC cell lines. However, unlike McGregor et al. (14), we had followed a 3-day 0.5 μM 5-aza-CdR treatment with a 24-h 5-aza-CdR plus ATRA treatment. Because ATRA is required for the transcriptional activation of the RAR-β2 promoter (25), the addition of ATRA after 5-aza-CdR pretreatment in our studies can explain our different findings.

The degree of methylation of 11 sites in the RAR-β2 promoter varied among the cell lines, which showed RAR-β2 methylation. Several (UMSCC17B, UMSCC22B, UMSCC35, UMSCC38) exhibited a high-density hypermethylated promoter, whereas other cell lines (MDA886Ln, ScCC/Y1, 1186, and TR146) had a partially methylated promoter. Nonetheless, there was a significant correlation between promoter hypermethylation and RAR-β2 mRNA expression level. Clones with partially methylated promoter expressed some RAR-β2, possibly due to the presence of unmethylated alleles.

Two (UMSCC10A and UMSCC19) of the eight HNSCC cell lines with unmethylated RAR-β2 failed to express RAR-β2 mRNA. The mechanisms of this silencing is not clear; however, it may be the result of histone deacetylation because induction of histone acetylation of the RAR-β2 promoter P2 using a histone deacetylase inhibitor (trichostatin A) could reactivate RAR-β2 transcription even from a methylated promoter in some breast cancer cells (59). RAR-β2 was expressed in one of our HNSCC cell lines (MDA886Ln), although the promoter was methylated, indicating that the chromatin structure was permissive for gene expression despite the methylation. 5-Aza-CdR pretreatment had failed to restore RAR-β2 inducibility by ATRA in the UMSCC17A and 17B cells. However, we found that the histone deacetylase inhibitor sodium butyrate can restore RAR-β2 expression in these cells. (Gillenwater and Lotan, unpublished data).

Several of the HNSCC cell lines (UMSCC17A, 17B, 22A, 22B, and 38), which we studied here had been reported to have lost one allele of chromosome 3p on which the RAR-β gene is located at 3p24 (29). RAR-β2 is methylated in all of these cell lines, and they fail to express RAR-β2 mRNA. These cells are an example of suppression of gene expression by a genetic loss of one allele and an epigenetic silencing of the remaining one, which has been reported for several tumor suppressor genes (30–32). These data suggest that the suppression of the RAR-β gene is an important event in the process in head and neck carcinogenesis.

A statistically significant inverse correlation was found between the methylation of the RAR-β gene and p53 mutation. Cells with wild-type p53 gene had a hypermethylated RAR-β2 gene. If RAR-β2 has a tumor suppressor function, then the silencing of its expression may be sufficient to enhance malignant transformation without the need to mutate p53. Previously, an inverse correlation has been reported between methylation of p16 and mutation of the p53 gene in colon carcinomas (60). Unfortunately, an analysis of 10 primary tumors had failed to confirm the cell line results. The reason for the difference between findings with cell lines and in vivo tumors is not clear.

Previous studies have demonstrated that transfection of exogenous RAR-β2 into head and neck cancer cells increased cell response to ATRA-induced growth inhibition and apoptosis (23). Therefore, it was plausible to ask whether restoration of RAR-β2 expression in HNSCC cell lines by pretreatment with 5-aza-CdR followed by 5-aza-CdR plus ATRA could restore sensitivity to growth inhibition or apoptosis. Because we found that all HNSCC cell lines were sensitive to 5-aza-CdR alone as
was reported also in other cancer cells (33, 38), we used this agent at a dose that causes 50% growth inhibition to determine whether ATRA added after 5-aza-CdR pretreatment under conditions that restore or increase RAR-β2 expression above the level observed in cells treated with ATRA alone or 5-aza-CdR alone would also enhance growth inhibition in some of the HNSCC cell lines as proposed previously (61). Indeed, we found that the combination of pretreatment with 5-aza-CdR and treatment with ATRA inhibited the growth of UMSCC22A, -22B, -38A, and 1483 cells more than each agent alone. In contrast, the effect of a similar treatment of UMSCC17A and 17B cells in which no increase in RAR-β2 was observed resulted in no or low enhanced activity over the single agent treatment. Several studies by others have demonstrated that 5-aza-CdR was an effective growth inhibitor as a single agent treatment. Several studies by others have demonstrated that 5-aza-CdR was an effective growth inhibitor as a single agent (33, 38), and a few studies suggested that this effect may be at least in part the result of the restoration of RAR-β2 expression (38, 44). Some clinical activity of 5-aza-CdR was also reported previously (34, 35). These results suggest but do not prove that the increase in RAR-β2 might be involved in the enhanced growth inhibition. However, many other genes that are modulated by 5-aza-CdR could be involved in the overall growth inhibitory effect observed. Nonetheless, the latter studies and our studies support the idea that the combination of low doses of 5-aza-CdR and ATRA might be developed into clinical trials with various malignancies.

REFERENCES


Hypermethylation of the Retinoic Acid Receptor-β2 Gene in Head and Neck Carcinogenesis

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