• ESOPHAGEAL CANCER •

Downregulation of retinoic acid receptor-*b₂* expression is linked to aberrant methylation in esophageal squamous cell carcinoma cell lines

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Supported by China Key Program on Basic Research, G1998051021, the Chinese Hi-tech R&D program, 2001AA231041, and National Science Foundation of China, 30170519
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Received: 2003-12-10   **Accepted:** 2004-01-08

Abstract

AIM: To study the role of hypermethylation in the loss of retinoic acid receptor *b₂* (*RARb₂*) in esophageal squamous cell carcinoma (ESCC).

METHODS: The role of hypermethylation in *RARb₂* gene silencing in 6 ESCC cell lines was determined by methylation-specific PCR (MSP), and its methylation status was compared with *RARb₂* mRNA expression by RT-PCR. The MSP results were confirmed by bisulfite sequencing of *RARb₂* promoter regions.

RESULTS: Methylation was detected in 4 of the 6 cell lines, and the expression of *RARb₂* was markedly downregulated in 3 of the 4 methylated cell lines. The expression of *RARb₂* was restored in one *RARb₂*-downregulated cell line with the partial demethylation of promoter region of *RARb₂* after 5-aza-2'-deoxycytidine (5-aza-dc) treatment.

CONCLUSION: The methylation of the 5' region may play an important role in the downregulation of *RARb₂* in some ESCC cell lines, suggesting that multiple mechanisms contribute to the loss of *RARb₂* expression in ESCC cell lines. This study may have clinical applications for treatment and prevention of ESCC.

INTRODUCTION

Retinoids, the important factors in modulating cell growth, differentiation, apoptosis and suppressing carcinogenesis *in vitro* and *in vivo*, are a group of natural and synthetic vitamin A analogs. In many animal models, such as cancers of skin, lung, prostate and esophagus, retinoids could suppress or reverse epithelial carcinogenesis[1]. The effects of retinoids are mainly mediated through two classes of nuclear receptors: the retinoic acid receptors (RARs) and retinoid X receptors (RXRs) which belong to a steroid/thyroid hormone-receptor superfamily. Each of them is composed of 3 subtypes (α, β and γ), of which *RARb* is expressed as three isoforms: *b1*, *b2* and *b4*[2]. Most human cells express *RARb2* as the major isoform. Many studies have confirmed that altered expression or function of the retinoid receptors may be related to malignant transformation of human cells[3,4].

RARb2 was decreased in many tumors, including lung carcinoma, breast cancer and esophageal cancers[5-7]. A study of esophageal cancer demonstrated that loss of *RARb* expression was an early event associated with esophageal carcinogenesis and the status of squamous differentiation[6], however the mechanisms underlying the inactivation of *RARb2* expression in cancer, especially the esophageal cancer, have not been well known yet. In breast cancer, no mutation or polymorphism was detected within the *b*RARE promoter, which was located in the promoter regions of target genes, and the lack of correlation between *RARb2* expression and LOH at chromosome 3p24 was demonstrated in esophageal cancer[8,9]. The *RARb2* promoter was characterized by an island, which was located in the 5'-untranslated region, and increasing data showed that aberrant methylation of the CpG islands in tumors was associated with transcriptional repression of tumor suppressor genes, such as *p*16 in esophageal cancer[10]. To clarify the epigenetic mechanism of *RARb2* loss or reduction in ESCC cell lines, we analyzed the methylation status of the *RARb2* promoter region by MSP assay and bisulfite sequencing. Our results indicated that downregulation of *RARb2* expression was associated with the aberrant methylation of the CpG islands in some ESCC cell lines, but not in others.

MATERIALS AND METHODS

Reagents and chemicals
5-aza-dc (Sigma, St. Louis, MO) was dissolved in pure ethanol at a stock concentration of 2 mM, and stored in aliquots at -70 ℃.

ESCC cell cultures and treatments
Six cell lines were used in this study: KYSE180, KYSE410, KYSE450, KYSE510, COLO680N, and TE12 were purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH). Each of the cell lines was cultured in either 90% RPMI 1640 medium (Invitrogen, Carlsbad, CA) or 45% RPMI 1640/45% Ham's F-12 medium (Invitrogen) with 100 mL/L fetal bovine serum at 37 ℃ containing 50 mL/L CO₂. In the demethylation experiment, the cells with lower *RARb₂* expression were treated with 5-aza-dc at a final concentration of 5 μmol/L for 96 h. The growth medium with or without the drug was changed every 24 h.

DNA preparation

The esophageal cell cultures were digested with trypsin-EDTA before collection. Genomic DNA from cell lines was extracted by proteinase-K digestion and phenol/chloroform extraction as described previously[11]. DNA was dissolved in TE buffer and stored at -20 ℃.

RNA isolation and semi-quantitative RT-PCR

Total RNAs were isolated from ESCC cell lines with Trizol reagent (Invitrogen) according to the manufactures' instructions. RNA quality was assessed with agarose gel electrophoresis and spectrophotometric analysis. Ten μg of total RNA of each sample was treated with 2 μL DNase I (10 units/μL, Promega, Madison, WI), 1 μL LRNasin (40 units/μL, Promega) at 37 ℃ for 15 min to remove contaminated DNA. First strand cDNA was reversely transcribed from 5 μg of total RNAs using SuperScript™ first-strand synthesis system for RT-PCR II kit (Invitrogen) at 42 ℃ for 80 min and 0.5-1 μL aliquots of the cDNA was then subjected to RT-PCR. The primers used for the amplification of *RARb2* were as follows: sense 5'-ATCGATGCCAATACTGTCGA-3', antisense 5'-GACTCGATGGTCAGCACTG-3' [8]. As, *GAPDH* was used as an internal control to ensure quality and quantity of cDNA for each RT-PCR. A product with 241 bp was generated, and PCR products were analyzed on 20g/L agarose gels. The PCR assay for each sample was repeated at least twice.

Methylation-specific PCR

Bisulfite modification of genomic DNA from primary esophageal cancer cell lines was carried out essentially as described previously[12]. Briefly, 2 μg of genomic DNA was denatured with freshly prepared NaOH (final concentration 0.3 mol/L) for 15 min in a 37 ℃ water bath, 30 μL of freshly prepared 10 mmol/L hydroquinone (Sigma) and 520 μL of freshly prepared 3.6 mol/L sodium bisulfite (Sigma) at pH 5 were added and mixed. The samples were then incubated under mineral oil for 16 h at 50 ℃. The modified DNA was purified with a Wizard DNA clean-up system (Promega). After purification, the samples were treated with NaOH (0.3 M) for 15 min at 37 ℃. The DNA was ethanol-precipitated and re-suspended in water for MSP analysis.

The bisulfite-induced changes affecting unmethylated (U) and methylated (M) alleles were detected by nested PCR. The primers for the first round PCR were as follows: sense 5'-AAGTGAGTTGTTTAGAGGTAGGAGGG3', and antisense 5'-CCTATAATTAATCCAAATAATCATTTACC-3' [13]. The amplification was performed under the following modified conditions: pre-denaturation at 95 ℃ for 5 min; 35 amplification cycles (denature at 95 ℃ for 15 s; annealing at 53 ℃ for 15 s; and extension at 72 ℃ for 30 s) and final extension at 72 ℃ for 5 min for the first round of PCR. The PCR products were diluted (1:10 dilution) and used in the second round of PCR with the primers: forward 5'-TTGAGAATGTGAGTGATTTGA-3', reverse 5'-AACCAATCCAACCAAAACAA-3' for the unmethylated sequence; and forward 5'-TCGAGAACGCG-AGCGATTCG-3', reverse 5'-GACCAATCCAACCGAAA-CGA-3' for the methylated sequences[14]. The amplification was performed under following conditions: pre-denaturation at 95 ℃ for 5 min; 35 amplification cycles (denature at 95 ℃ for 15 s; annealing at 52 ℃ for 15 s; and extension at 72 ℃ for 15 s for U-primers) and pre-denaturation at 95 ℃ for 5 min; 20 amplification cycles (denature at 95 ℃ for 15 s; annealing at 62 ℃ for 15 s; and extension at 72 ℃ for 15 s M-primers). DNA from normal lymphocytes was used as negative control for methylated alleles, and placental DNA treated *in vitro* with SssI methyltransferase (New England biolabs, Beverly, MA) was used as positive control for methylated genes. The product of 146 bp was generated, and the PCR products were analyzed on 25g/L agarose gels and photographed. The controls without DNA were performed in each set of PCR, and MSP assay for each sample was repeated at least twice.

Bisulfite sequencing

The PCR products amplified with primers specific either for the methylated or for the unmethylated DNA were purified and cloned into the pMD-T vector (Takara, Dalian, China) according to the manufacture's protocol. Recombinants were transformed into *Escherichia coli*. Plasmid DNA was isolated and integrated PCR fragments were verified by enzyme digestion and PCR. The cloned PCR fragments were further analyzed by automated sequencing (Bioasia, Shanghai, China).

RESULTS

Expression of RARb2 in ESCC cell lines

The expression of the *RARb2* mRNA was detected in the six ESCC cell lines by RT-PCR. Figure 1 shows representative examples of RT-PCR and MSP results. Markedly downregulation of *RARb2* expression was observed in the cell lines KYSE410, KYSE510 and COLO680N as shown in Figure 1A.

Methylation analysis of the RARb2 promoter region

Using MSP method, both the methylated and unmethylated alleles were found in the four cell lines except KYSE180 and TE12 in which only the unmethylated alleles were present (Figure 1B). The quantitation of the unmethylated or the methylated products varied in different cell lines. To confirm the reliability of the nested MSP method, PCR products amplified with primers specific either for the methylated (cell lines KYSE410) or for the unmethylated (cell line KYSE180) DNA were cloned and sequenced. Bisulfite sequencing of 4 individual clones of PCR products from cell line KYSE180 revealed no methylation of CpGs within the promoter region, whereas the methylated products showed dense methylation within the CpG islands in cell line KYSE410 (Figure 2).

Association between RARb2 promoter methylation and downregulation of its expression in ESCC cell lines

The reduction of *RARb2* mRNA expression was observed in three of the four cell lines that had partially methylated alleles, however the another one cell line showed strong *RARb2* gene expression, which suggesting that the aberrant methylation might play other roles except leading to gene silencing.

Restoration of RARb2 expression after 5-aza-dc treatment

To confirm whether *RARb2* promoter methylation could be further linked to downregulation of its expression, the cancer cell lines which showed evidently decreased expression of *RARb2* and cell line KYSE180 without methylated alleles were treated with the demethylating agent (5-aza-dc). To determine the effects of demethylating agent, we analyzed the methylation status in KYSE410 cells after 5-aza-dc treatment, and partial demethylation was detected by MSP as shown in Figure 3A. In addition, the MSP product was still observed after treatment because 5-aza-dc only inhibited methylation of newly synthesized DNA. The level of *RARb2* mRNA was not changed in cell line KYSE180 before and after demethylating treament. Among the three cell lines where *RARb2* genes were methylated, the treatment led to up-regulation of *RARb2* mRNA expression only in cell line KYSE410 (Figure 3B). The other two cell lines showed no detectable re-expression of *RARb2* mRNA after 5-aza-dc treatment (data not shown). These data indicated that *RARb2* promoter methylation was closely associated with downregulation of *RARb2* expression in some ESCC cell lines.
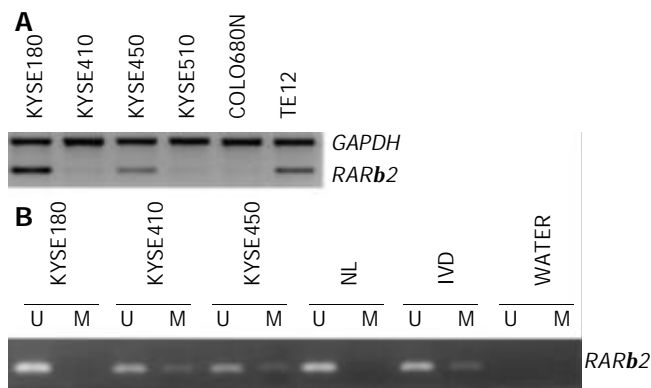
Figure 1 *RAR*β*2* expression and methylation in ESCC cell lines. A: RT-PCR analysis of *RARb2* expression, *GAPDH* was used as internal control. B: MSP analysis of *RARb2* promoter methylation status, (U) lanes and (M) lanes represent amplification of unmethylated and methylated alleles, respectively. *In vitro* methylated DNA (IVD) and normal human peripheral lymphocytes (NL) serve as positive and negative methylation controls, respectively.
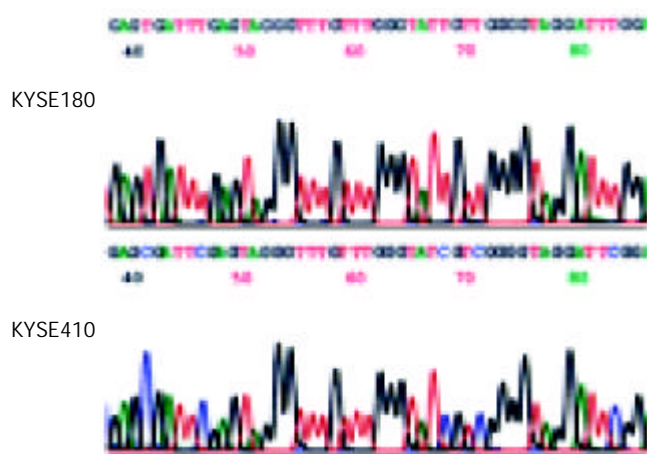

Figure 2 Sodium bisulfite sequencing of *RARb2* in the cell lines that were found to include only unmethylated alleles (cell line KYSE180) or partial methylated alleles (cell line KYSE410) by MSP.
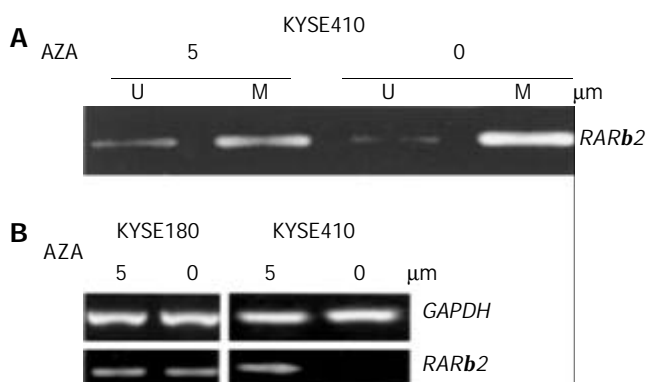

Figure 3 Treatment with the indicated concentrations of 5-aza-dc induced demethylation and *RARb2* mRNA expression in ESCC cell lines. A: MSP analysis shows partial demethylation of the *RARb2* promoter region after 5-aza-dc treatment. B: RT-PCR analysis of *RARb2* mRNA expression before and after 5-aza-dc treatment in cell lines that were found to have either positive (cell line KYSE180) or reduced (cell line KYSE410) baseline *RARb2* expression, *GAPDH* was used as internal control.

DISCUSSION

There were two major *RARb2* antineoplastic mechanisms in breast cancer cell lines, which were RA-dependent and RA-independent apoptosis[15,16], however little was known how *RARb2* performed its anticancer activity. A growing evidence indicated that *RARb2* was required for the growth inhibitory action of RA. In *RARb2*-negative cancer cells, RA-induced growth inhibition and decreased tumorigenicity could be restored by expression of *RARb2*; on the contrary, in *RARb2*-positive cancer cells, RA effects could be abolished by inhibition of *RARb2* expression[17-19]. Another study indicated that not only expression but also up-regulation of *RARb* was linked to retinoid sensitivity in esophageal cancer cell lines[20], and our previous study also suggested that RA resistance in EC109 cells paralleled with the loss of *RARb2* inducibility[21]. A clinical trial using N-4-(ethoxycarbophenyl) retinamide, a synthetic retinoid, demonstrated that cancer incidence among the treatment groups with severe esophageal dysplasia was decreased by 43.2% compared with the placebo group[22], whereas this was not the case for recent clinical trials of 13-cis retinoic acid in the treatment of advanced esophageal cancer patients, for which an explanation was that the loss of *RARb* expression could lead to the resistance to treatment with 13-cis RA in esophageal cancer[23,24]. Recent study suggested that hypermethylation of *RARb* might play an important role in the early stage of esophageal squamous cell carcinogenesis[25]. However, the mechanisms of *RARb2* suppression remained largely unknown in esophageal cancer.

Genetic and epigenetic mechanisms may be involved in progressive decrease in *RARb2* mRNA expression during esophageal carcinogenesis. *RARb* was localized on chromosome 3p24, and LOH occurred frequently on chromosome arm 3p in many cancers, including esophageal cancer[2]. However, no correlation was observed between the expression of *RARb* and the LOH on chromosome 3p24 in ESCC; moreover, no mutations or other genetic alterations were found in different cell lines without *RARb2* mRNA expression[9,26,27]. Thus, other mechanisms for *RARb2* suppression should be considered. DNA methylation, which led to the inactivation of all kinds of essential genes, such as *HLA class I, E-cadherin, FHIT* and *p*16, was an important mechanism for gene silencing or suppressing in ESCC[10,28-30]. In addition, Arapshian *et al*[31] concluded that methylation of the RARE region might be particularly important in *RARb2* suppression. Data presented in this report demonstrated that epigenetic silencing of the *RARb2* gene promoter might be an important event, and that downregulation of *RARb2* expression was closely associated with *RARb2* gene promoter methylation in some ESCC cell lines. In our study, of the three *RARb2*-downregulated cell lines, only the one poorly differentiated cell line showed evidently re-expression of *RARb2* with the partial demethylation of its promoter region, indicating that repression was, at least in part, mediated by methylation in the poorly differentiated cell lines, and multiple mechanisms were involved in the gene silencing in the other two cell lines. Our results suggested that a relationship between methylation status and decreased *RARb2* expression only existed in some differentiated ESCC cell lines or ESCC. Previous study has demonstrated that for breast carcinoma a relationship between methylation status and decreased *RARb2* expression in breast cancer was found only in invasive grade II lesions[13]. There was mounting evidence that suggested epigenetic modifications might be a mechanism responsible for the lack of *RARb2* expression in many epithelial cancers or cancer cell lines, such as breast cancer, colon cancer and prostate cancer[8,13,32,33]. Taken together the further analysis of the cytogenetic data of the cell lines, we found that there was

no deletion in 3p in the cell line exhibiting re-expression of *RARb2*. We therefore speculate that methylation may be at least one of the major mechanisms resulting in loss of *RARb2* expression in such cell lines. It has been reported that in ESCC cell lines without any structural alteration, hypermethylation at CpG sites was the important mechanism for transcription inactivation of the *FHIT* gene[30]. Given that the *RARb2* gene is recognized as a putative tumor suppressor gene, restoration of its function may have implications for both cancer therapy and prevention.

Notely, demethylation could only lead to the restoration of *RARb2* expression in 1/3 ESCC cell lines without 3p deletion, but not in the other cell lines with 3p deletion, indicating that there were other mechanisms, such as deletion, involved in the loss of *RARb2* expression in ESCC cell lines. It was reported that differential function of coactivators and corepressors might determine the level of *RARb* induction that might mediate retinoid action in colon cancer cells[34]. In lung cancer cell lines, loss of *RARb* gene expression was linked to aberrant histone H3 acetylation, so the aberrant acetylation status might have effects on *RARb2* expression[35]. In addition, orphan receptor COUP-TF have been identified to play a key role in modulating *RARb* expression and retinoid sensitivity in cancer cells[36]. Thus, multiple mechanisms may contribute to the loss of *RARb2*.

In conclusion, this study not only shows a high frequency of methylation of *RARb2* promoter region but also provides the first evidence that downregulation of *RARb2* is closely associated with DNA methylation in ESCC cell lines. These findings will be helpful to further understand the mechanisms underlying tumor progression in ESCC, and subsequently improve the prevention and treatment.

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