Methylation and Silencing of the Retinoic Acid Receptor-β2 Gene in Breast Cancer

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Background: **A growing body of evidence supports the hypotheses that the retinoic acid receptor β2 (RAR-β2) gene is a tumor suppressor gene and that the chemopreventive effects of retinoids are due to induction of RAR-β2. RAR-β2 expression is reduced in many malignant tumors, and we examined whether methylation of RAR-β2 could be responsible for this silencing.** ***Methods:*** **RAR-β2 expression was studied by reverse transcription–polymerase chain reaction (RT–PCR) analysis in eight breast cancer cell lines that were either treated with the demethylating agent 5-aza-2′-deoxycytidine and subsequently with all-*trans*-retinoic acid (ATRA) or left untreated. Sodium bisulfite genomic sequencing was used to determine the locations of 5-methylcytosines in the RAR-β2 genes of three of these cell lines. In 16 breast cancer biopsy specimens and non-neoplastic breast tissue, methylation-specific PCR was used to determine the methylation status of RAR-β2, and, in 13 of the specimens, RT–PCR analysis was used to detect RAR-β2 expression.** ***Results:*** **Cell lines SK-BR-3, T-47D, ZR-75-1, and MCF7 exhibited expression of RAR-β2 only after demethylation and treatment with ATRA. The first exon expressed in the RAR-β2 transcript was methylated in cell lines ZR-75-1 and SK-BR-3. Six breast cancer specimens showed methylation in the same region of the gene. No expression of RAR-β2 was found in any grade III lesion. An inverse association between methylation and gene expression was found in all grade II lesions. The RAR-β2 gene from non-neoplastic breast tissue was unmethylated and expressed.** ***Conclusions:*** **Methylation of the RAR-β2 gene may be an initial step in breast carcinogenesis; treatment of cancer patients with demethylating agents followed by retinoic acid may offer a new therapeutic modality. [J Natl Cancer Inst 2000;92:826–32]**

The incidence of breast cancer has been increasing since the 1940s, making it the most common malignancy among women in the developed countries *(1)*. Worldwide, 10%–15% of all women will be diagnosed with breast cancer during their lifetimes. The identification of novel approaches for the prevention and treatment of breast cancer is urgently needed. An appealing approach, in theory, for the prevention of breast cancer is the use of retinoids, structural and functional analogues of vitamin A *(2)*.

Retinoids are known to possess antiproliferative, differentiative, immunomodulatory, and apoptosis-inducing properties. Certain retinoids suppress premalignant oral lesions and prevent the development of second primary cancers among patients with head and neck and lung cancers *(3,4)*. Anzano et al. *(5,6)* showed that 9-*cis*-retinoic acid, alone or in combination with tamoxifen or raloxifene, is a potent inhibitor of mammary carcinogenesis induced by *N*-nitroso-*N*-methylurea in Sprague-Dawley rats.

The regulation of cell growth and differentiation of normal, premalignant, and malignant cells by retinoids is thought to result from their direct and indirect effects on gene expression. These effects are mediated by the nuclear receptors, retinoic acid receptor (RAR)-α, -β, and -γ and retinoid X receptor (RXR)-α, -β, and -γ, which are ligand-activated transcription factors and members of the steroid hormone receptor superfamily. Retinoid receptors activate transcription in a ligand-dependent manner by binding as RAR/RXR heterodimers or RXR homodimers to retinoic acid response elements (RAREs) located in the promoter regions of target genes.

One of the target genes of retinoid receptors is RAR-β *(7)*, which encodes four transcripts. RAR-β2 messenger RNA (mRNA)is transcribed from a promoter in front of exon 3, the first exon expressed in the RAR-β2 transcript *(8)*. In the RAR-β2 promoter region, the retinoic acid-response element β-RARE mediates retinoic acid-induced RAR-β2 gene expression in many different cell types *(9)*.

It has been found *(10–14)* that RAR-β2 mRNA is not expressed in a number of malignant tumors, including lung carcinoma, squamous cell carcinoma of the head and neck, and breast cancer cell lines. These findings support the concept that the specific loss of RAR-β2 mRNA expression may be an important event during tumorigenesis. This hypothesis is supported by the observation that introduction of RAR-β protein into retinoic acid-insensitive breast cancer cell lines restored retinoic acid responsiveness (growth inhibition and induction of apoptosis) *(15)*. A functional study *(16)* demonstrated that RAR-β2, like the tumor suppressor gene p53, can inhibit oncogene-induced focus formation. The RAR-β2 gene was shown to be derepressed in normal, senescing mammary epithelial cells but to be repressed in malignant cell lines *(10)*. Furthermore, RAR-β2 has been shown to function as a tumor suppressor gene in epidermoid lung carcinoma cells *(14)* and in gynecologic cancer cells *(17)*.

We reported that RAR-β mRNA expression is absent in breast cancer and morphologically normal adjacent tissue but is always present in normal breast tissue distant from the cancer *(18)*. Sequencing of DNA extracted from paraffin-embedded tumor tissue of the corresponding breast cancer specimens did not reveal any mutations in the RAREs. This

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See "Notes" following "References."

observation suggests that another mechanism is responsible for repression of RAR-β.

Methylation of DNA inhibits transcription, and the strong inhibitory effect of 5-methylcytosine in mammalian promoter regions suggests that methylation interferes with transcription initiation [*(19)* and references therein]. Methylation has been observed to have a similar effect when it occurs in normally unmethylated GC-rich regions in the promoter and in coding regions of tumor suppressor genes, including p16 *(20,21)*, VHL *(22)*, BRCA1 *(23)*, and hMLH1 *(24)*, and the E-cadherin invasion-suppressor gene *(25)*. To our knowledge, Côté and Momparler *(26)* were the first to demonstrate that aberrant methylation could be involved in RAR-β suppression in the DLD-1 colon carcinoma cell line.

In this study, we examined whether gene inactivation by methylation could be responsible for repression of RAR-β2 in breast cancer cell lines and whether the RAR-β2 gene is methylated in breast tissue from patients with breast cancer.

MATERIALS AND METHODS

Chemicals and Reagents

All-*trans*-retinoic acid (ATRA), 5-aza-2′-deoxycytidine (Aza-CdR), Igepal CA-630, and phenylmethylsulfonyl fluoride were from Sigma Chemical Co. (St. Louis, MO). For all experiments that used ATRA or Aza-CdR, 1 m*M* solutions were prepared in dimethyl sulfoxide and diluted in culture medium (*see below*).

Breast Tissue Specimens

Biopsy specimens from 16 patients with invasive ductal carcinoma of the breast (five grade II and 11 grade III lesions) *(27)* were obtained immediately after resection of the breast or lumpectomy. Specimens were brought to the pathologist, and a part of the tissue was placed into liquid nitrogen and stored at −80 °C until lyophilization. We also used an identically treated sample of normal breast tissue from a patient who had undergone a mastectomy. This study was approved by the Institutional Review Board of the Department of Obstetrics and Gynecology of the University of Innsbruck, Austria.

Cell Culture and Treatment

Human breast cancer cell lines were obtained from the following sources: MCF7 (M. Lippman, Georgetown University Medical Center, Washington, DC); Hs 578T and BT-20 (G. Buehring, University of California, Berkeley); T-47D, MDA-MB-231, HBL-100, and SK-BR-3 (N. Hynes, Friedrich Miescher Institute, Basel, Switzerland); and ZR-75-1 (R. King, University of Surrey, Guildford, U.K.). They were cultured as described elsewhere *(11)* in modified Eagle medium containing 10% fetal calf serum. Cells were incubated either for 5 days with 2.5 μ*M* Aza-CdR, for 24 hours with 1 μ*M* ATRA, or for 5 days with 2.5 μ*M* Aza-CdR followed by 24 hours with 1 μ*M* ATRA or left untreated. They were detached with trypsin (0.05%)–EDTA (0.02%) in Dulbecco's phosphate-buffered saline (PBS), washed, and pelleted.

Reverse Transcription–Polymerase Chain Reaction

Total RNA from breast tissues and cells was isolated as described previously *(11)*. We used the GeneAmp® EZ rTth RNA PCR Kit (The Perkin-Elmer Corp., Norwalk, CT) for the reverse transcription–polymerase chain reaction (RT–PCR) reactions. The RT–PCR reactions were carried out in a total volume of 50 μL and contained 5 μg of RNA, 10 μL of GeneAmp 5× EZ buffer (i.e., 250 m*M* *N,N*-bis[2-hydroxyethyl]glycine [pH 8.2], 575 m*M* potassium acetate, and 8% glycerol [vol/vol]), 300 μ*M* of each deoxynucleoside triphosphate (dNTP), 2.5 m*M* of manganese acetate, 0.45 μ*M* of each primer, 5 U of rTth DNA polymerase, and 40 μL water. (Perkin-Elmer rTth DNA polymerase is a recombinant, thermostable DNA polymerase encoded by a modified form of the *Thermus thermophilus* DNA polymerase gene and possesses both reverse transcriptase and DNA polymerase activity.)

Samples were processed in a Perkin-Elmer 9600 GeneAmp thermocycling system under the following conditions: 40 minutes at 61 °C for the reverse transcriptase reaction and a 90-second denaturation step at 94 °C followed by 18 amplification cycles (45 seconds at 94 °C for denaturation, 30 seconds at 61 °C for primer annealing, and 1 minute at 72 °C for primer extension), five cycles as above but with the extension step lengthened to 75 seconds, and another five cycles as above but with an extension step of 90 seconds. PCR products were analyzed on 2% agarose gels. Amplification of RAR-β2 used primer sequences that spanned three introns (5′-AGA GTT TGA TGG AGT TGG GTG GAC-3′ [exon 3: nucleotide, i.e., nt, 376 to nt 399] for amplification of the sense strand and 5′-GCT GGC AGA GTG AAG GGA AAG TTT-3′ [exon 6: nt 127 to nt 150] for amplification of the antisense strand) and produced a single polydeoxynucleotide of 721 base pairs (bp). Primers for the low-density lipoprotein (LDL) receptor (5′-CAA TGT CTC ACC AAG CTC TG-3′ [sense] and 5′-TCT GTC TCG AGG GGT AGC TG-3′ [antisense]) produced a polydeoxynucleotide of 276 bp that was used as a loading control.

DNA Isolation From Tissues and Cultured Cells

Genomic DNA samples from breast cancer cell lines were prepared by use of the QIAmp Blood Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Genomic DNA from lyophilized breast cancer tissue and normal breast tissue was isolated by use of the QIAmp Tissue Kit (Qiagen).

Sodium Bisulfite Genomic Sequencing

Bisulfite treatment of single-stranded DNA converts unmethylated cytosines to uracil but does not affect methylated cytosines. Uracil is recognized as thymine by *Taq* polymerase and, hence, the product of the PCR will contain cytosines only at positions where 5-methylcytosines occurred in the starting template DNA. Genomic DNA was treated as described by Zeschnigk et al. *(28)*. An 8-μL aliquot of 3 *M* NaOH was added to a 4-μg sample of DNA (in 70 μL of water). The solution was incubated for 15 minutes at 37 °C, denatured at 95 °C for 3 minutes, and immediately cooled on ice. The denatured DNA solution was mixed with 1 mL of bisulfite reagent (freshly prepared by dissolving 8.1 g of sodium bisulfite into 15 mL of water, adding 1 mL of 40 m*M* hydroquinone, and adjusting the pH to 5.0 with 3 *M* NaOH), overlaid with mineral oil, and incubated in the dark for 16 hours at 55 °C. The DNA was recovered by adsorbing to 5 μL of glassmilk (GeneClean III Kit; Bio 101, Inc., Vista, CA) and eluting with 100 μL of water. For desulfonation, 11 μL of 3 *M* NaOH was added, and the samples were incubated for 15 minutes at 37 °C and neutralized by adding 110 μL of 6 *M* ammonium acetate (pH 7.0). The DNA was precipitated with ethanol, washed in 70% ethanol, dried, and resuspended in 20 μL of water. The concentration of the bisulfite-treated DNA was estimated with DNA DipSticks™ (Invitrogen Corp., San Diego, CA).

The 635-bp region containing part of the promoter and first exon expressed in the RAR-β2 transcript was amplified from the bisulfite-modified DNA with two rounds of PCR by use of nested primers specific to the bisulfite-modified sequence of this region. The primers were as follows:

Primer 1 (nt −414 to nt −386)—5′-GTA TAG AGG AAT TTA AAG TGT GGG TTG GG-3′; primer 2 (nt +268 to nt +296)—5′-CCT ATA ATT AAT CCA AAT AAT CAT TTA CC-3′; primer 3 (nt −357 to nt −329)—5′-GTA GG(C/T) GGA ATA TTG TTT TTT AAG TTA AG-3′; and primer 4 (nt +251 to nt +278)—5′-AAT CAT TTA CCA TTT TCC AAA CTT ACT C-3′.

Primers 1 and 2 were used in the first round of amplification in a reaction volume of 50 μL, by use of 50–100 ng of bisulfite-treated DNA as template. PCR mixtures further included 5 μL of 10× PCR buffer (i.e., 100 m*M* Tris–HCl [pH 8.3], 500 m*M* KCl, and 15 m*M* $MgCl_2$; Roche Molecular Biochemicals, Vienna, Austria), 100 μ*M* of each dNTP, 600 n*M* of each primer, and 1.25 U of *Taq* polymerase. PCR was carried out under the following conditions: 94 °C for 5 minutes followed by 35 amplification cycles (15 seconds at 94 °C for denaturation, 15 seconds at 55 °C for primer annealing, and 30 seconds at 72 °C for extension) and a final extension reaction (5 minutes at 72 °C). Of the first-round PCR product, 2 μL was used as template for the second round of PCR that used primers 3 and 4 under the same conditions. The 635-bp PCR products were analyzed on 2% agarose gels and sequenced by use of the LI-COR 4200 automatic sequencer (LI-COR, Lincoln, NE).

Methylation-Specific PCR Assay

This assay takes advantage of DNA sequence differences between methylated and unmethylated alleles after bisulfite modification. Reacting DNA with sodium bisulfite converts all unmethylated cytosines to uracil, which is recognized as thymine by *Taq* polymerase, but does not affect methylated cytosines. Amplification with primers specific for methylated or unmethylated DNA discriminates between methylated and unmethylated DNA. It is a simple and fast way of surveying multiple samples to detect methylation of cytosines in the region of

interest: a 146-bp fragment in the 5′ region of the first exon expressed in the RAR-β2 transcript, a region that is methylated in both ZR-75-1 and SK-BR-3.

In a first round of PCR, a DNA fragment ranging from nt +18 to nt +296 was amplified with primers specific for bisulfite-modified DNA: 5′-AAG TGA GTT GTT TAG AGG TAG GAG GG-3′ (sense) and 5′-CCT ATA ATT AAT CCA AAT AAT CAT TTA CC-3′ (antisense). PCR mixtures included, in a total volume of 50 μL, 2–10 ng DNA, 5 μL of 10× PCR buffer, 100 μ*M* of each dNTP, 600 n*M* of each primer, and 1.25 U of *Taq* polymerase. PCR was carried out under the following conditions: 94 °C for 5 minutes followed by 35 amplification cycles (15 seconds at 94 °C for denaturation, 15 seconds at 53 °C for annealing, and 30 seconds at 72 °C for extension) and a final extension incubation of 5 minutes at 72 °C. The DNA fragment resulting from this amplification was then further amplified by methylation-specific PCR (MSP), by use of primers designed *(29)* to detect methylation in the region of interest. MSP primer sequences were 5′-TTG AGA ATG TGA GTG ATT TGA-3′ (sense, nt +105 to nt +124) and 5′-AAC CAA TCC AAC CAA AAC AA-3′ (antisense, nt +231 to nt +250) for amplification of the modified, unmethylated sequence and 5′-TCG AGA ACG CGA GCG ATT CG-3′ (sense, nt +105 to nt +124) and 5′-GAC CAA TCC AAC CGA AAC GA-3′ (antisense, nt +231 to nt +250) for amplification of the modified, methylated sequence. MSP was performed with reaction mixtures of the same composition as those above, under the following conditions: 94 °C for 1 minute followed by 35 amplification cycles (30 seconds at 94 °C for denaturation, 30 seconds at 55 °C for annealing, and 30 seconds at 72 °C for extension) and a final extension incubation of 5 minutes at 72 °C. PCR products were analyzed on 1.5% agarose gels.

Nuclear Protein Extracts and Western Blot Analysis

Tissues were homogenized gently in a Dounce homogenizer with ice-cold buffer A (i.e., 10 m*M* Tris–HCl [pH 7.4], 250 m*M* sucrose, and 3 m*M* $MgCl_2$), and nuclei were collected by centrifugation (800*g* for 20 minutes). Nuclear pellets were solubilized by incubation in buffer B (i.e., 50 m*M* HEPES [pH 7.4], 500 m*M* NaCl, 1% Igepal CA-630, and 100 μg/mL phenylmethylsulfonyl fluoride) for 15 minutes on ice. The extracts were centrifuged (10 000*g* for 2 minutes); the resulting supernatants are referred to as the nuclear extract. All of the above procedures were carried out at 4 °C. Protein quantitation was performed by use of the Coomassie protein assay reagent (Pierce Chemical Co., Rockford, IL).

Electrophoresis of 50-μg samples of nuclear extracts was carried out on 12% polyacrylamide gels containing sodium dodecyl sulfate. After electrophoresis, the gels were blotted to a Hybond ECL™ nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, U.K.). The membrane was blocked in 5% nonfat dry milk (Bio-Rad Laboratories, Hercules, CA) in PBS with 0.1% Tween 20 (PBS-T) for 1 hour at room temperature, followed by incubation for 90 minutes with a 10-mL solution of RAR-β primary polyclonal antibody raised against a C-terminal RAR-β epitope (sc-552X; Santa Cruz Biotechnology, Santa Cruz, CA) (diluted 1 : 500 in PBS-T). After four 5-minute washes with PBS-T, the membrane was incubated for 30 minutes with peroxidase-conjugated anti-rabbit secondary antibody (diluted 1 : 1000 in PBS-T). The blot was visualized by use of ECL western blotting chemiluminescent detection reagent (Amersham Pharmacia Biotech).

Results

Expression of RAR-β2 mRNA by RT–PCR After Demethylation

To evaluate expression of the RAR-β2 gene, the level of the corresponding mRNA was estimated by RT–PCR in eight breast cancer cell lines. Cells from each cell line were untreated, incubated with the demethylating agent Aza-CdR (2.5 μ*M*) for 5 days, incubated with 1 μ*M* ATRA for 24 hours, or incubated with 2.5 μ*M* Aza-CdR for 5 days followed by 1 μ*M* ATRA for 24 hours. Cell lines SK-BR-3, T-47D, ZR-75-1, and MCF7 showed expression of RAR-β2 mRNA only after demethylation and treatment with ATRA. Cell line MDA-MB-231 expressed the gene constitutively, with an increase in the level of expression after demethylation and treatment with ATRA. Cell lines Hs 578T and HBL-100 expressed the gene constitutively but showed no increase in expression after demethylation. Cell line BT-20 showed no expression of the RAR-β2 gene, regardless of treatment (Fig. 1). RAR-β2 transcripts were detected in a control specimen of non-neoplastic breast tissue.

5-Methylcytosine Analysis of the 5′ Promoter–Exon Region of the RAR-β2 Gene

We used the sodium bisulfite genomic sequencing method to identify 5-methylcytosine residues in the 5′-promoter–exon

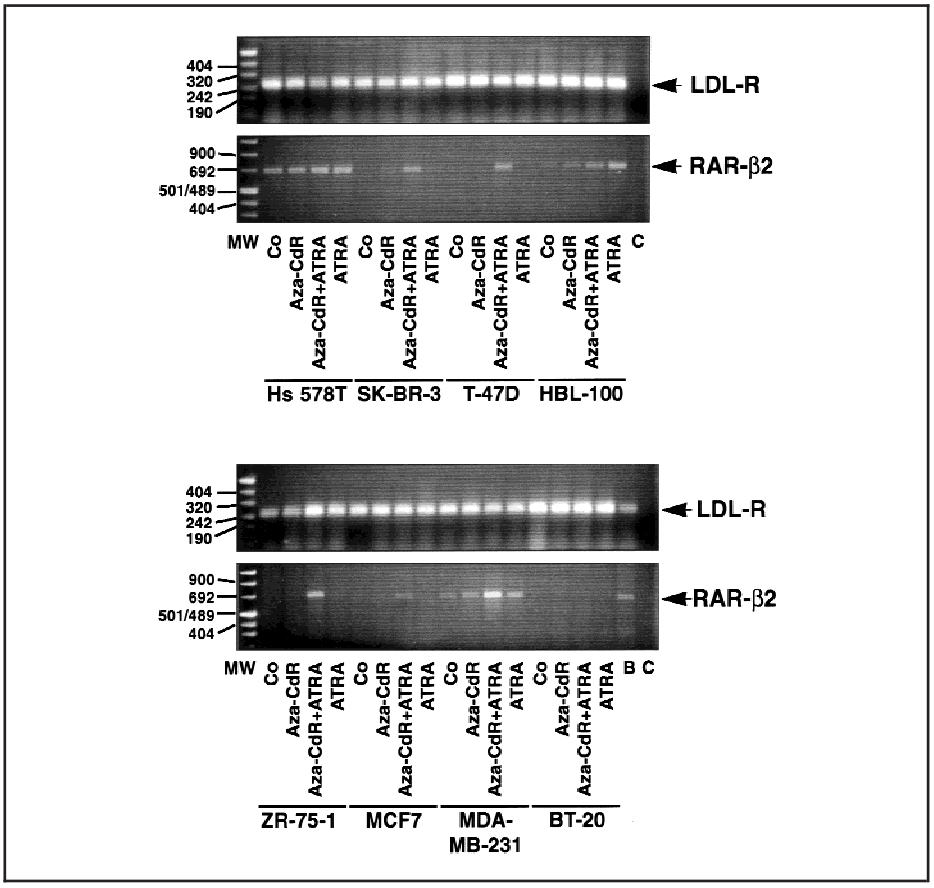

Fig. 1. Retinoic acid receptor (RAR)-β2 messenger RNA (mRNA) expression in breast cancer cell lines. Cells from cell lines Hs 578T, SK-BR-3, T-47D, HBL-100, ZR-75-1, MCF7, MDA-MB-231, and BT-20 were left untreated (**Co lanes**), incubated for 5 days with the demethylating agent 5-aza-2′-deoxycytidine (Aza-CdR) (**Aza-CdR lanes**), incubated for 5 days with Aza-CdR and for 24 hours with all-*trans*-retinoic acid (ATRA) (**Aza-CdR + ATRA lanes**), or incubated with ATRA alone for 24 hours (**ATRA lanes**). Total RNA was isolated as described previously *(11)* and amplified by the reverse transcription–polymerase chain reaction (PCR) procedure as described in the text. PCR products (RAR-β2 complementary DNA [cDNA] [721 base pairs (bp)] and low-density lipoprotein receptor [LDL-R] cDNA [276 bp, used as a loading control]) were analyzed on 2% agarose gels. Controls were non-neoplastic breast tissue from a mastectomy patient (**lane B**) and mock reverse-transcribed RNA (reverse transcriptase omitted) (**C lanes**). Size markers are shown (with their sizes in bp) in the **left-most lane** (**MW**) of each gel.

regions of the RAR-β2 genes in three breast cancer cell lines. We designed primers to analyze a 635-bp region containing part of the promoter region and the first exon expressed in the RAR-β2 transcript and found 21 and 13 residues of 5-methylcytosine in the genes from cell lines ZR-75-1 and SK-BR-3, respectively (Fig. 2). Methylation in both the promoter region and in the first exon expressed in the RAR-β2 transcript was observed in the DNA of ZR-75-1 cells. It was observed only in the first exon expressed in the RAR-β2 transcript of the SK-BR-3 cells. No methylation was observed in either the part of the promoter region analyzed or the first transcribed exon of the RAR-β2 gene from cell line Hs 578T. The GenBank accession number for the promoter is emb/X56849/HSRARBP, and the accession number for the RAR-β2 first transcribed exon is gb/M96016/HUMRAR21.

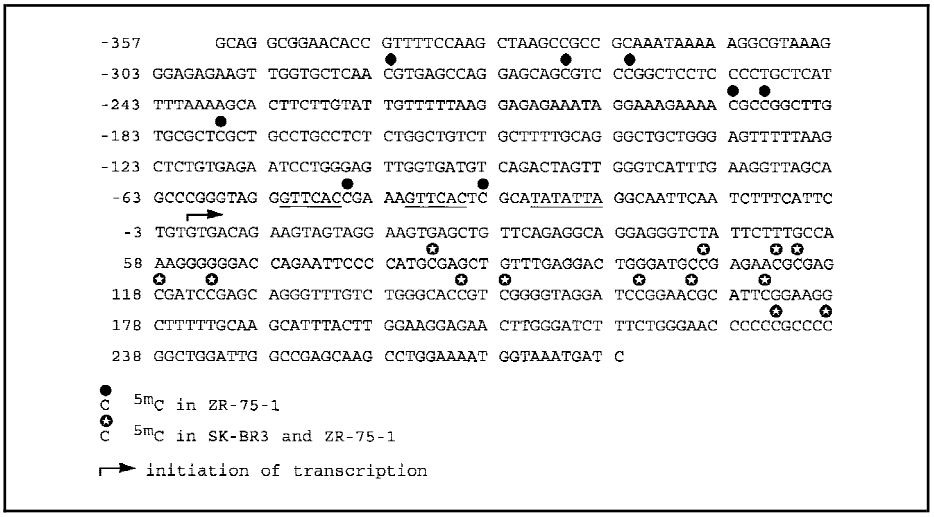

Fig. 2. DNA sequence of the promoter–exon region of the retinoic acid receptor (RAR)-β2 gene. Genomic DNA was isolated from three breast cancer cell lines both before and after incubation of the cells with demethylating agent 5-aza-2′-deoxycytidine (Aza-CdR). The isolated DNA was reacted with bisulfite as described in the text, amplified by the polymerase chain reaction, and sequenced twice. The sequences of the region of the RAR-β gene from nucleotide (nt) –357 to nt 278 from Aza-CdR-treated and untreated cells were compared to determine where 5-methycytosine (^{5m}C) occurs in the native genome. The transcription start site is indicated by an **arrow.** The two RAR elements and the TATA box are **underlined.** The positions of the 5-methylcytosines are indicated by **filled circles** for cell line ZR-75-1 and by **filled circles with stars** for cell lines ZR-75-1 and SK-BR-3. Cell line Hs 578T showed no methylation.

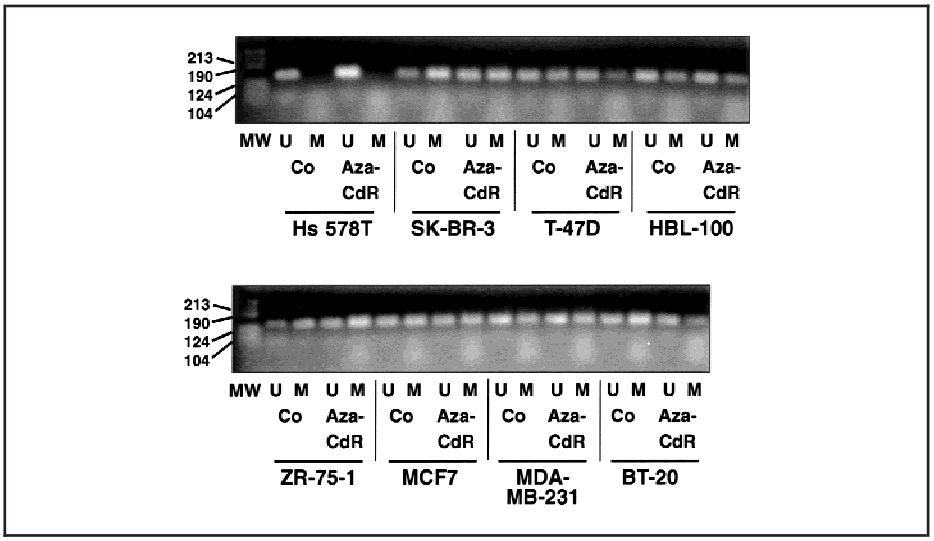

Fig 3. Methylation-specific polymerase chain reaction (MSP) of human breast cancer cell lines. Cells from cell lines Hs 578T, SK-BR-3, T-47D, HBL-100, ZR-75-1, MCF7, MDA-MB-231, and BT-20 were left untreated (**Co lanes**) or incubated for 5 days with the demethylating agent 5-aza-2′-deoxycytidine (Aza-CdR) (**Aza-CdR lanes**), treated with bisulfite as described in the text, and amplified by the polymerase chain reaction by use of primers specific for methylated (**M lanes**) and unmethylated (**U lanes**) DNA. The presence of a 146-base-pair (bp) polymerase chain reaction product in lanes marked **U** indicates that the retinoic acid receptor (RAR)-β2 gene is unmethylated in the corresponding DNA; product in lanes marked **M** indicates that RAR-β2 is methylated. Size markers are shown (with their sizes in bp) in the **left-most lane** (**MW**) of each gel.

Methylation in Breast Cell Lines and Breast Cancer Specimens

We used MSP to examine breast cancer cell lines for methylation in the 146-bp fragment in the 5′ region of the first exon expressed in the RAR-β2 transcript, which is methylated in both ZR-75-1 and SK-BR-3. Of the eight cell lines examined, only Hs 578T, which expresses RAR-β2 constitutively (Fig. 1), showed no methylation. In all of the other seven cell lines, our results revealed a mixture of methylated and unmethylated sequences in the relevant region of the RAR-β2 gene. Aza-CdR was not able to demethylate completely the 146-bp region that we examined in any of the cell lines studied (Fig. 3).

We studied 16 primary breast cancer biopsy specimens (five grade II and 11 grade III lesions). Methylation in the 5′ region of the RAR-β2 gene was seen in six tumors. Unmethylated DNA was present in all 16 specimens. The one nonneoplastic breast tissue specimen that we examined showed no methylation (Fig. 4, A).

To assess whether methylation of the RAR-β2 gene is associated with its expression, RT–PCR of RAR-β2 was performed on 13 of the 16 breast cancer specimens whose methylation status we determined (five from grade II and eight from grade III lesions) and on the nonneoplastic breast tissue specimen. The three grade II tumor specimens that were shown to be unmethylated expressed RAR-β2 mRNA; the two that were shown to be methylated did not. None of the specimens from grade III lesions expressed this message (Fig. 4, B). RAR-β2 transcript was also detected in nonneoplastic breast tissue.

Western blot analysis was used to detect expression of RAR-β protein in one breast cancer specimen and in the nonneoplastic breast tissue specimen. By use of an antibody against a C-terminal RAR-β epitope for detection of proteins after electrophoresis, a protein of molecular mass ~48.8 kd, which corresponds to the molecular mass of RAR-β2, was iden-

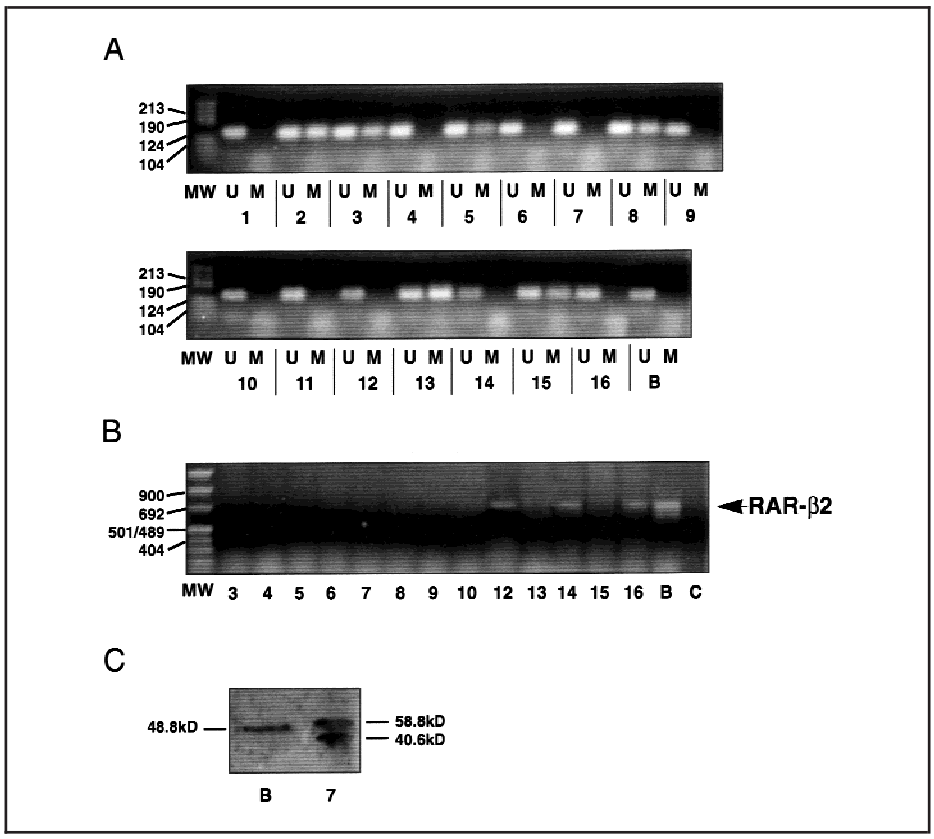

Fig. 4. Methylation-specific polymerase chain reaction (MSP) analysis of DNA from primary breast cancer specimens and non-neoplastic breast tissue and expression of RNA and protein. **Panel A:** Genomic DNA from 16 primary breast cancer specimens was isolated and treated with bisulfite as described in the text and amplified by the polymerase chain reaction (PCR) by use of primers specific for methylated (**M**) and unmethylated (**U**) DNA. Specimens 1–11 were grade III lesions; specimens 12–16 were grade II lesions. **B** denotes the non-neoplastic breast tissue specimen. The presence of a 146-base-pair (bp) PCR product in lanes marked **U** indicates that the 5′ region of the retinoic acid receptor (RAR)-β2 gene is unmethylated in the corresponding specimen; product in lanes marked **M** indicates that RAR-β2 is methylated. Size markers are shown (with their sizes in bp) in the **left-most lane** (**MW**) of each gel. **Panel B:** expression of RNA. **Lanes 3–16** correspond to breast cancer specimens analyzed for methylation in the experiment shown in panel A. RNA was extracted, and reverse transcription–PCR analysis was used to detect RAR-β2 messenger RNA (721 bp). Controls were the non-neoplastic breast cancer specimen (**lane B**) and mock reverse-transcribed RNA (reverse transcriptase omitted) (**lane C**). Size markers are shown (with their sizes in bp) (**MW lane**). **Panel C:** western blot analysis of nuclear extracts. Nuclear extracts of non-neoplastic breast tissue (**lane B**) and breast cancer specimen **7** (corresponding to tissue 7 in panels A and B) were prepared as described in the text. After electrophoresis on a 12% polyacrylamide gel containing sodium dodecyl sulfate, the samples were blotted onto a nitrocellulose membrane. The blots were incubated with polyclonal antibody to a C-terminal epitope of RAR-β protein and then with a peroxidase-conjugated secondary antibody. Detection was by chemiluminescence. kD = kilodalton.

tified in the specimen of non-neoplastic breast tissue. The same antibody detected RAR-β proteins with molecular masses of ~58.8 and ~40.6 kd in the nuclear extract of one breast cancer specimen.

DISCUSSION

A lack of RAR-β2 mRNA expression has been observed in solid tumor cells, including lung carcinoma, squamous cell carcinoma, and breast cancer *(10,12–14,18,30).* A growing body of evidence supports the hypotheses that the RAR-β2 gene is a tumor suppressor gene *(14,15,31)* and that the chemopreventive effects of retinoids are due to induction of RAR-β2 *(32).* The mechanism of RAR-β2 suppression remains unclear. Côté et al. *(26,29)* were the first to suggest that methylation of the RAR-β2 gene may be the mechanism by which the gene's expression is silenced in a colon carcinoma cell line.

Transcriptional repression by DNA methylation can be mediated through a sequence-independent process that involves changes in chromatin structure and histone acetylation levels: Binding of the transcriptional repressor MeCP2 to methylated DNA is followed by recruitment of a complex containing a transcriptional corepressor and a histone deacetylase. Deacetylation of histones is associated with reduced levels of transcription, perhaps by allowing tighter nucleosomal packing [*(19)* and references therein]. When the RXR–RAR heterodimer binds to β-RARE, the chromatin structure undergoes dynamic, reversible changes in—but also around—the RAR-β2 promoter *(33).* Minucci et al. *(34)* reported that the histone deacetylase inhibitor trichostatin A substantially increases the accessibility of the restriction endonuclease *Sma*I to the RAR-β2 promoter.

Our findings demonstrated several points about the relationship between RAR-β2 gene methylation and suppression of this gene in breast cancer: 1) Methylation occurred in the promoter and the first exon expressed in the RAR-β2 transcript in seven tested cell lines (the one exception was Hs 578T, a breast cancer cell line that, according to our results, expresses RAR-β2 constitutively); 2) methylation of this region could also be observed in six of 16 breast cancer specimens; 3) RAR-β2 expression could be restored (in four of five cell lines that did not express the gene constitutively) or increased (in one of three cell lines that expressed the gene constitutively) by treating breast cancer cell lines with the demethylating agent Aza-CdR followed by treatment with retinoic acid; and 4) there is a common GC-rich region in the first exon expressed in the RAR-β2 transcript.

The methylation region that is common to the two cell lines in which we detected methylation of RAR-β2 is in the first transcribed exon, not in the promoter. Another example of a tumor suppressor gene where methylation does not occur mainly in the promoter region is the p16/CDKN2A gene, in which exon 2 frequently becomes methylated *de novo* and gene expression is suppressed *(35).* Methylation of tumor suppressor genes—and, therefore, escape from processes that lead the cells to physiologic turnover (e.g., apoptosis)—seems to be one of the initial events in carcinogenesis. It was reported *(36)* that senescence of human mammary epithelial cells that demonstrate an extended period of growth (compared with the main part of the normal mammary epithelial cells) is associated with aberrant methylation and loss of expression of the tumor suppressor gene p16. The cause of *de novo* methylation during carcinogenesis remains unclear. In 1993, Wu et al.

(37) showed that constitutive overexpression of an exogenous mouse DNA methyltransferase gene in mouse 3T3 fibroblasts results in a marked increase in overall DNA methylation, which is accompanied by tumorigenic transformation of these cells.

Jones and Laird *(19)* have hypothesized that methylation could act as a third pathway (in addition to intragenic mutations and loss of chromosomal material) for full inactivation of a tumor suppressor gene. We used MSP to study methylation in the common GC-rich region in exon 1 of the RAR-β gene and showed that only cell line Hs 578T, which expresses the RAR-β2 gene constitutively, had only unmethylated alleles. In all of the other cell lines studied, both methylated and unmethylated alleles could be found. This observation suggests that biallelic inactivation is required for RAR-β2 suppression, as was also shown to be true for the hMLH1 gene *(24,38)*. In addition, it has been reported *(39)* that, in 48% of breast cancer specimens, loss of heterozygosity was detected at chromosomal region 3p24, a locus that includes the region coding for RAR-β2. Assuming that methylation is a prerequisite for histone deacetylation in the RAR-β2 gene, our finding that there is a common GC-rich region in the first exon expressed in the RAR-β2 transcript and that methylation of that region suppresses expression of the gene supports the hypothesis *(19)* that methylation of the RAR-β2 gene could lead to its allelic loss.

We found that demethylation restores RAR-β2 expression but that Aza-CdR treatment did not completely demethylate the GC-rich region of RAR-β2 exon 1. Cameron et al. *(40)* showed that minimal demethylation by low-dose Aza-CdR resulted in slight gene reactivation. The presence of nonmalignant cells (i.e., fibroblasts, blood cells, and some normal epithelial cells) in the tumor biopsy specimens that we studied may account for the presence of unmethylated DNA.

Although, in view of a previous study *(18)*, the bulk of our data suggests that methylation of RAR-β2 is a common event during carcinogenesis in the breast, several observations complicate this interpretation. In the BT-20 cell line, which is strongly methylated, Aza-CdR could not restore RAR-β2 expression. A relationship between methylation status and RAR-β2 expression was found only in grade II lesions. Only tumors having unmethylated RAR-β2 expressed the gene. In grade III lesions, expression of RAR-β2 was never observed, regardless of methylation status. One explanation for repression of RAR-β2 expression in grade III lesions could be the involvement of Nur77. Overexpression of this gene has been found to be associated with a loss of RAR-β2 inducibility and retinoic acid resistance in lung cancer cells *(41)*.

Western blot analysis of protein expression by use of an antibody raised against a C-terminal RAR-β epitope demonstrated expression of the ~48.8-kd RAR-β2 protein only in a non-neoplastic breast specimen, whereas the breast cancer specimen we examined demonstrated two apparently different RAR-β proteins with molecular masses of ~40.6 and ~58.8 kd. The quite surprising detection of two RAR-β proteins in an RAR-β2 mRNA-negative breast cancer cell line was recently reported by Sommer et al. *(42)*: They identified the ~40.6-kd species as RAR-β4 and hypothesized that this isoform functions as a dominant-negative repressor of RAR-mediated growth suppression.

Our data, in combination with those from a previous study *(18)*, provide only indirect evidence that methylation and subsequent loss of RAR-β2 expression are early steps in breast carcinogenesis. Direct evidence for our hypothesis should be provided by an ongoing study that is examining normal breast tissue adjacent to and distant from the cancerous lesion and breast cancer specimens by use of a combination of laser-assisted cell picking and real-time PCR *(43)*.

Our results show that the 5′ region of the RAR-β2 gene is methylated in many breast cancer cell lines and tumors and that treatment of the cell lines with the demethylating agent Aza-CdR restores inducibility of RAR-β2 by ATRA. In animal models, the administration of demethylating agents has shown a strong chemopreventive effect *(44,45)*. Knowledge of the methylation status of the RAR-β2 gene may facilitate chemoprevention by selective re-expression of the gene in preneoplastic tissue by treatment with ATRA after administration of a demethylating agent.

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NOTES

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