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Research paper

Demethylation by 5-aza-2′-deoxycytidine of specific 5-methylcytosine sites in the promoter region of the retinoic acid receptor β gene in human colon carcinoma cells

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The role of retinoic acid receptor β (RARβ), a putative tumor suppressor gene, in the development of colon malignancy still remains to be clarified. We reported previously that the expression of RARβ in DLD-1 human colon adenocarcinoma cells was silenced by DNA methylation at the level of the promoter region (*Anti-Cancer Drugs* 1997; 8: 56). In addition, we observed that RARβ expression could be activated by the hypomethylating action of 5-aza-2′-deoxycytidine (5-Aza-CdR). In this report we have identified, by sequencing of bisulfite-modified DNA of DLD-1 colon tumor cells, the specific 5-methylcytosine positions in the region of −46 to +251 bp from the transcription start site of RARβ$_2$. We observed that 5-Aza-CdR treatment demethylated these specific sites. Based on this sequence data, specific primers for the methylation-specific PCR (MSP) assay were designed to discriminate methylated from unmethylated CpG sites in the promoter region of RARβ. This assay confirmed the changes in the methylation status of the RARβ gene in DLD-1 colon tumor cells before and after treatment with 5-Aza-CdR. The methylation status of the promoter region of the RARβ gene was also examined in primary human colon adenocarcinomas using the MSP assay. Six of the 14 colon tumor samples showed signs of hypermethylation of this gene. The MSP assay for RARβ may be a useful tool to clarify the role of DNA methylation for this gene in colon tumorigenesis.
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Key words: 5-Aza-2′-deoxycytidine, colon cancer, DNA methylation, retinoic acid receptor β.

Introduction

The tumorigenesis of colon cancer results from the stepwise accumulation of genetic changes.[1] Genetic susceptibility to a high risk of colon cancer was reported in patients with a mutation in the APC gene[2] or the mismatch repair gene.[3] Mutations in the K-*ras* gene occur frequently in patients with colon carcinoma and is a poor prognostic factor for this disease.[4] Deletions and mutations of the p53 gene in colon cancer have also been reported.[5]

Epigenetic changes that involve DNA methylation may also be implicated in colon tumorigenesis. The importance of DNA methylation with respect to the control of expression of tumor suppressor genes is currently under active investigation.[6,7] Methylation of CpG islands in the promoter region of these genes results in the silencing of gene transcription.[9,10] In colon cancer the expression of the p16 tumor suppressor gene[11] and hMLH1 mismatch repair gene[12] were shown to be silenced by DNA methylation. It is interesting to note that colorectal tumors that show microsatellite instability due to defects in mismatch repair genes show a higher incidence of hypermethylation of the p16 gene and thrombospondin-1 angiogenesis inhibitor gene.[13] In other types of tumors, hypermethylation was observed for the tumor suppressor genes such as the p16 cyclin-dependent kinase inhibitor gene,[14,15] Rb gene,[16] VHL gene,[17] BRCA-1 gene[18] and mammary-derived growth inhibitor gene,[19] the invasion suppressor gene , E-cadherin,[20] and the repair gene, methylguanine-DNA methyltransferase.[21]

Retinoids can produce profound effects on the growth and differentiation of normal and malignant cells.[22] The diverse cellular effects of retinoids are mediated by their binding to nuclear retinoic acid receptors (RARs) which act as transcription factors that bind to RAR response elements (RAREs) located in the promoter region to modulate gene expression.[23] Houle *et al.*[24] proposed that RARβ functions as a tumor suppressor gene in lung tumorigenesis. Several studies support this hypothesis. RARβ maps to the

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short arm of chromosome 3, a region which is frequently deleted in lung cancer.[25,26] RARβ is poorly or not expressed in a number of malignant tumors, including lung,[27,28] head and neck,[29,30] breast,[31] cervix,[32] and ovary.[33] Gene transfer of RARβ cDNA into tumor cells that do not express this gene results in a suppression of tumor growth.[24,34] In addition, transgenic mice expressing an antisense to RARβ show an enhanced development of tumors.[35] These results suggest that the loss of RARβ expression may contribute to the tumor formation. The role of this nuclear receptor in the development of colon cancer still remains to be clarified.

We reported previously that RARβ was not expressed in DLD-1 human colon adenocarcinoma cells; the presence of 5-methylcytosine residues in its promoter region was detected by Southern blot analysis.[36] In addition, we observed that RARβ expression could be activated by 5-aza-2′-deoxycytidine (5-Aza-CdR), a potent inhibitor of DNA methylation. A synergistic antiproliferative interaction between this cytosine nucleoside analog and all-*trans* retinoic acid was observed against the DLD-1 colon tumor cells.[37]

The objective of this study was to define the methylation status of CpG sites in the promoter region of the RARβ gene of the DLD-1 colon carcinoma cells and to determine if treatment of these tumor cells with 5-Aza-CdR could demethylate these sites. We sequenced the bisulfite-modified DNA to identify the methylation sites[38] in the promoter region of the RARβ gene. The second objective was to design primers for the MSP reaction[39] and to analyze the methylation status of the RARβ promoter region in samples of primary human colon carcinoma.

Materials and methods

Cell line and tumor samples

The human DLD-1 colon adenocarcinoma cell line was obtained from the American Type Tissue Culture Collection (Rockville, MD). The cell line was maintained in culture in RPMI 1640 (Canadian Life Technologies, Burlington, Ontario, Canada) with 10% fetal calf serum from Wisent (St Bruno, Québec, Canada) in a 5% CO_2 incubator at 37°C. For the study of demethylation, the cell line DLD-1 was treated with 0.5 μM 5-Aza-CdR (Decitabine) from Pharmachemie (Haarlem, The Netherlands) for 3 days. Genomic DNA of the DLD-1 cell line was extracted, before and after 5-Aza-CdR treatment, with the Stratagene (La Jolla, CA) DNA isolation kit as specified by the manufacturer. Genomic DNA from colon tumors and normal colon tissue from patients was prepared by a standard procedure using proteinase K digestion and phenol-chloroform extraction.

Bisulfite reaction

Bisulfite treatment of single-stranded DNA converts all unmethylated, but not methylated, cytosines to uracil. The bisulfite reaction was carried out according to the procedure of Frommer *et al.*[38] and Herman *et al.*[39] Briefly, 2 μg of genomic DNA was fragmented by enzymatic digestion with 10 U of *Mbo*II (Canadian Life Technologies) and by passing through a 27 gauge needle before denaturation with freshly prepared NaOH at a final concentration of 0.3 M for 15 min at 75°C. Freshly prepared sodium bisulfite (pH 5.0) (Sigma, Oakville, Ontario, Canada) and hydroquinone (Sigma) were added to each denatured DNA sample to a final concentration of 4.2 M and 0.5 mM, respectively, and incubated under mineral oil in the dark at 55°C for 4 h. The bisulfite salts were removed by the Prep-A-Gene DNA purification kit (BioRad Laboratories, Mississauga, Ontario, Canada) according to the manufacturer's instructions. The DNA was desulfonated by addition of freshly prepared NaOH at a final concentration of 0.3 M, incubated at 37°C for 15 min, neutralized with 3 M ammonium acetate (pH 7.0) and precipitated with 3 volumes of ethanol. The DNA was resuspended in TE (pH 7.5).

PCR amplification and primers

A 629 bp portion of the 5′ promoter region of the RARβ gene (Figure 1) was amplified by PCR for the analysis of the specific sites of DNA methylation by sequencing. The specific RARβ primers used to amplify bisulfite-converted top strand DNA were as follows: sense 5′-GAG AAG TTG GTG TTT AAT GTG AGT T-3′, position 544–568; antisense 5′- CAT AAA TTA TAA CAA ACA AAC CAA C-3′, position 1172–1148 of the human $RAR\beta_2$ promoter region[40] (GenBank accession no. X56849). The underlined bases indicate the position of modification by the bisulfite treatment.

PCR amplifications were performed in 50 μl reaction mixtures containing 5 ng bisulfite-treated DNA, 100 μM dNTPs, 0.2 μM primers, 1 mM $MgCl_2$, 8% glycerol, 1× buffer and 1.25 U of ID-Proof Taq DNA polymerase (ID-Labs, London, Ontario, Canada) in a thermal minicycler (MJ Research, Watertown, MA) under the following conditions: 94°C for 2 min; 94°C for 30 s, 50°C for 30 s, 72°C for 1 min ×35 cycles; and 72°C for 5 min. PCR fragments were extracted

from agarose gel and purified with the QIAEX II agarose gel extraction kit (Qiagen, Mississauga, Ontario, Canada).

DNA cloning and sequencing

The 629 bp PCR fragment (100 ng) was cloned into pCR2.1-TOPO vector according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Plasmid DNA was purified using the Qiagen Plasmid Mini kit, and sequenced in both orientations using the AutoRead 1000 sequencing kit and ALF automatic sequencer (Pharmacia Biotech, Baie d'Urfe, Québec, Canada).

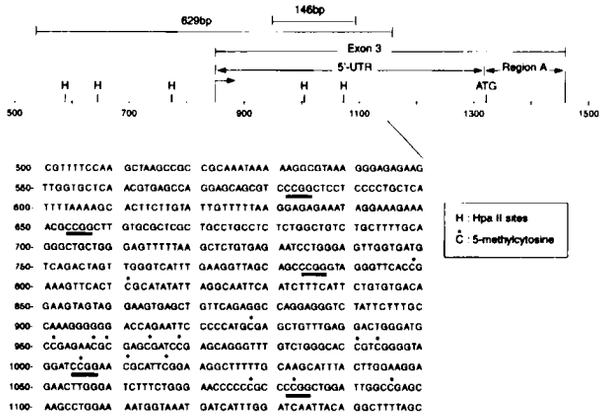

Figure 1. Genomic map and DNA sequence of the promoter–exon region of the RARβ_2 gene (GenBank accession no. X56849[40]). The transcription start site (position 844) is indicated by a broken arrow. Exon 3 (position 844–1469) contains the 5'-UTR and the coding region A of the RARβ_2 protein. The ATG start codon is at position 1314–1316. The HpaII sites are indicated by the letter H and are double underlined in the indicated sequence. The indicated 629 bp fragment (position 544–1172) represents the region that was sequenced after the bisulfite treatment. The 146 bp fragment (position 950–1095) shown on the map was the region analyzed for CpG methylation by the MSP assay. The 5-methylcytosine positions are indicated by an asterisk over the C in the indicated sequence. The two RAREs are at positions 792–797 and 803–808, respectively. The TATA box is located at position 814–820. The Sp1 element is located at position 1074–1081.

Methylation-specific PCR (MSP) assay

Recently, Herman *et al.*[39] developed an efficient PCR-based assay, MSP, that takes advantage of DNA sequence differences between methylated and unmethylated alleles after bisulfite modification. Primer pairs for the 5' promoter region of RARβ (RARβ-M and RARβ-U; Table 1) were used for methylation-specific PCR. They were designed to analyze the methylation sites in a 146 bp fragment in the 5' promoter region of the RARβ_2 gene (Figure 1), from position 950–969 (RARβ-M) and 950–970 (RARβ-U) for sense primers to position 1095–1076 for antisense primers. The PCR mixtures included 2–10 ng of bisulfite-modified DNA, 1 × PCR buffer, $MgCl_2$ (1.0 mM), 8% glycerol, dNTPs (100 μM), primer pairs (0.2 μM) and 1.25 U of ID-Proof Taq DNA polymerase in a final volume of 50 μl. Following an initial denaturation step of 2 min at 94°C, PCR was performed for 35 cycles (RARβ-M) or 40 cycles (RARβ-U) of 94°C for 30 s, 58°C (RARβ-M) or 50°C (RARβ-U) for 30 s and 72°C for 30 s followed by one cycle at 72°C for 5 min in a MJ Research thermal minicycler. Aliquots of the PCR samples were loaded on a 2% agarose gel for electrophoresis, stained with ethidium bromide and photographed.

Results

Identification of 5-methylcytosine sites in 5' promoter region of RARβ in DLD-1 colon carcinoma cells

Genomic DNA was isolated from a mass population of DLD-1 cells before and after treatment with 0.5 μM 5-Aza-CdR for 3 days. The DNA was fully denatured and treated with sodium bisulfite under conditions such that cytosine was converted to uracil whereas 5-

Table 1. PCR primers used for MSP assay of promoter region of RARβ

Primer set	Sense primer[a]	Antisense primer[a]
RARβ-W[b]	5'-CCGAGAACGCGAGCGATCCG-3'	5'-CCGCCCCGGCTGGATTGGCC-3'
RARβ-M	5'-**T**CGAGAACGCGAGCGA**TT**CG-3'	5'-G**A**CCAA**T**CCA**A**CCG**AAA**CG**A**-3'
RARβ-U	5'-**TT**GAGAA**T**G**T**GAG**T**GA**TTT**GA-3'	5'-**A**A**C**CAA**T**CCA**A**CC**A**AAA**C**A**A**-3'

[a]Sequence differences between modified primers and unmodified DNA are in boldface type and differences between methylated/modified and unmethylated/modified are underlined.
[b]W, unmodified or wild-type primers; M, methylated-specific primers; U, unmethylated-specific primers.

methylcytosine remains non-reactive (resistant to deamination). We mechanically sheared and restriction enzyme cleaved DNA from DLD-1 cells to facilitate denaturation by alkali. There exist different isoforms of the RARβ gene which are generated from the same gene by differential promoter usage and alternative splicing.[40,41] The 5′-UTR and start codon of the RARβ2 isoform are present in exon 3 (Figure 1). We designed primers to amplify a 629 bp fragment in the promoter-exon region of the $RAR\beta_2$ gene (position 544-1172) which was subcloned and sequenced.

Comparison of the sequence of this fragment from DLD-1 colon tumor cells, untreated and treated with 5-Aza-CdR, permitted the identification of the position of the 5-methylcytosine residues. Figure 1 shows the sequence from 500 to 1149 of the $RAR\beta_2$ gene and the position of the 5-methylcytosine residues as indicated by asterisks for the untreated cells. The region from 927 to 1095 contained 14 CpG sites which showed methylation, including two *Hpa*II sites (positions 1005 and 1082) that were methylated. Our sequencing data showed that the 5-Aza-CdR treatment of the DLD-1 tumor cells produced complete demethylation of all the methylated CpG sites shown in Figure 1. A methylated CpG site (position 798) was present between the two RARE consensus sequences (positions 792-797 and 803-808). The Sp1 binding element (position 1074-1081) contained a methylated CpG site at position 1077 of the DLD-1 tumor cells. There was also a methylated CpG site (position 810) at 4 bp upstream from the TATA box. These sites were demethylated by treatment with 0.5 μM 5-Aza-CdR for 3 days. There were regions where methylation was not detected (position 544-797), including three *Hpa*II sites (positions 581, 653 and 783) that were not methylated in the DLD-1 colon tumor cells.

Primer design for MSP assay

The identification of the positions of 5-methylcytosine in the 5′-UTR of the RARβ gene in DLD-1 colon tumor cells (Figure 1) permitted us to design primers for MSP analysis.[39] Primers were designed to discriminate between methylated and unmethylated alleles following bisulfite treatment of DNA. To accomplish this, primer sequences were chosen for a region containing frequent cytosines (to distinguish unmodified from modified DNA) and methylated CpG sites near the 3′-end of the primers (to provide maximal discrimination between methylated and unmethylated DNA). The sequence region chosen was contained within positions 950-1095 which is 106 bp downstream from the transcription start site (Figure 1). The wild-type sequence for this position is designated RARβ-W in Table 1. The primer pair that detects methylated DNA was designated as RARβ-M, whereas the pair that reveals unmethylated DNA was called RARβ-U (Table 1). These primers amplified a 146 bp fragment.

MSP analysis of RARβ in DLD-1 colon tumor cells and in primary colon adenocarcinomas

We tested the primers designed to evaluate the methylation status of the RARβ promoter in bisulfite-modified DNA from DLD-1 colon tumor cells (Figure 2A). The methylation status of the untreated DLD-1 colon tumor cells was confirmed by the presence of the predicted 146 bp amplified band with the RARβ-M primer set and by its absence when the RARβ-U primer set was used (Figure 2A, lane 1).

When these cells were treated with 0.5 μM 5-Aza-CdR for 3 days we detected the presence of a weak amplification with the RARβ-M primers (Figure 2A, lane 2). This result is in agreement with our previous Southern blot analysis of DNA methylation.[36] Amplification with the RARβ-U primer pair showed that this region was mostly demethylated by the presence of the 146 bp band (Figure 2A, lane 2). DNA not treated with bisulfite (unmodified) failed to amplify with either set of methylated or unmethylated specific primers (data not shown).

We examined the methylation status of the RARβ promoter in the bisulfite-modified genomic DNA from 14 patients with colon adenocarcinoma by MSP analysis (Figure 2B). Six of 14 patients with colon adenocarcinoma (nos 1, 2, 3 [weak band] 9, 10 [weak band] and 14) showed promoter hypermethylation of the RARβ, as revealed by the presence of a 146 bp band with the RARβ-M primers. The other eight patients had no RARβ hypermethylation, as revealed by the absence of an amplified fragment of 146 bp with RARβ-M primers. All these tumor samples showed the presence of a band when RARβ-U primers were used.

The MSP assay was performed on 11 samples of normal corresponding colon tissue (Figure 2C) as compared to tumor samples (Figure 2B). All the 11 normal samples showed a positive amplification of the 146 bp band with the RARβ-U primers. Three of the normal samples (nos 1, 3 and 14) showed a weak to very weak amplification with the RARβ-M primers. These results showed that eight out of 11 normal tissue samples did not display methylation of the RARβ gene with our MSP assay.

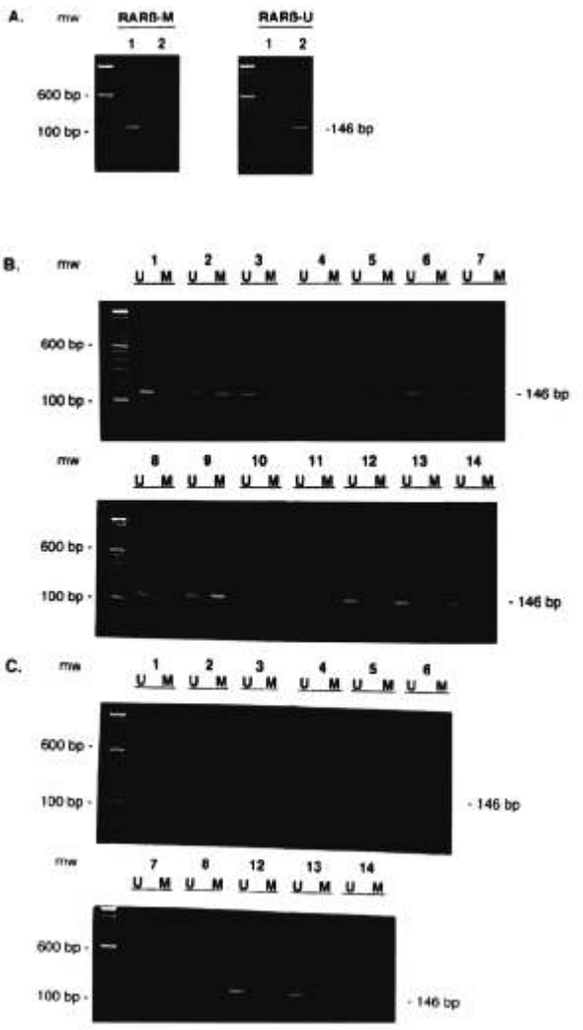

Figure 2. MSP analysis of the RARβ gene in colon cancer. All DNA samples were treated with bisulfite. (A) Amplification of a 146 bp fragment from DLD-1 colon tumor cells before (1) and after a 3 day treatment with 0.5 μM 5-Aza-CdR (2). Methylation-specific primer set (M, RARβ-M) and unmethylation-specific primer set (U, RARβ-U) were used for the MSP assay. Amplification of a 146 bp fragment from (B) colon adenocarcinomas (patients 1–14) and (C) normal corresponding colon tissue from the same patients. Molecular weight marker (mw).

Discussion

Hypermethylation of CpG islands in the promoter region of several tumor suppressor genes is associated with transcriptional inactivation.[11,15,17] This random methylation of CpG islands which are not methylated in normal cells leads to a progressive loss of expression of growth inhibitory genes and may be an important aspect of the multistep events leading to tumorigenesis.[6,7]

Retinoids can inhibit the *in vitro* growth and induce differentiation of tumors cell lines.[22] This action is due to the binding of this ligand to RARs which act as transcription factors.[23] RARβ, one of the receptors in this family, was proposed to function as a tumor suppressor gene.[24,34] In support of this hypothesis is the observation in lung cancer of the frequent loss of heterozygosity of the 3p chromosome region which contains the RARβ gene.[25,26] The frequent loss of expression of RARβ in lung and other cancers[27–33] is also in agreement with this proposal. It is possible that silencing of the RARβ gene present in the non-deleted allele occurs by mutation or methylation.

We have demonstrated previously the absence of RARβ mRNA expression in DLD-1 colon cells and the activation of its expression by 5-Aza-CdR treatment.[36] In addition, Southern blot analysis with methylation-sensitive enzymes indicated that the 5-Aza-CdR produced demethylation of the promoter region of the RARβ gene in these cells. We concluded from these data that methylation of the RARβ promoter region correlated inversely with its expression. Activation of RARβ expression provides one explanation for the synergistic antineoplastic action produced by 5-Aza-CdR in combination with all-*trans* retinoic acid on the DLD-1 tumor cells.[37]

In order to obtain a precise analysis of the methylated CpG sites, we subcloned and sequenced a 629 bp fragment of bisulfite-treated DNA[38] from the promoter region of RARβ of DLD-1 colon tumor cells. We observed that this 629 bp region contained 16 methylated CpG (Figure 1). 5-Aza-CdR treatment completely demethylated this region of DNA as shown by DNA sequencing. There was one methylated CpG site between the two RAREs and one methylated CpG site in the Sp 1 element. It is intriguing to speculate that the presence of these 5-methylcytosine residues interferes with the binding of RARβ to the RARE. CpG methylation was reported to interfere with the binding of the AP2 transcription factor.[42] The mechanism that triggered RARβ expression could be the direct result of the demethylation produced by 5-Aza-CdR or entail a more complicated mechanism involving induction of other genes that are suppressed by methylation. It is also possible that other events, such as chromatin structure or proteins that bind to methylated DNA, may play an important role in the control of the expression of RARβ.[10]

The precise position of methylated CpG sites in the promoter region of RARβ obtained by DNA sequencing was used to design methylation-specific and unmethylation-specific primers for the MSP reaction.[39]

The advantages of the MSP reaction are that it is simple, more sensitive than Southern blot analysis, specific and requires only small amounts of DNA. Using the RARβ-M primers (Table 1), we were able to confirm the presence of methylated CpG sites in the promoter region (position 950–1095) of the RARβ gene of DLD-1 tumor cells (Figure 2A). Treatment of these cells with 5-Aza-CdR resulted in an amplification of the predicted 146 bp PCR product with RARβ-U primers. We observed a weak amplification in the 5-Aza-CdR-treated cells with the RARβ-M primers, in agreement with our Southern blot analysis.[36] This minor band may have been due to a small fraction of cells that did not enter S phase during treatment or due to the remethylation of DNA after treatment with 5-Aza-CdR.[43] It is also possible, since we chose a concentration of 5-Aza-CdR that did not produce a 100% loss of clonogenicity, some tumor cells that survived this treatment may have retained the methylated RARβ gene.

We used the MSP analysis with the RARβ-M and RARβ-U primers to screen a tumor bank of human colon adenocarcinomas. Six of 14 tumor samples displayed the PCR pattern associated with hypermethylation of the RARβ promoter (Figure 2B). The amplification of a 146 bp fragment with RARβ-U primers for all these tumor samples probably represents the contamination of the colon tumor samples by normal tissue.

Methylation analysis was performed on 11 normal corresponding colon samples from these patients (Figure 2C). All these normal samples showed a predominance of the unmethylated RARβ gene. Three of the samples of normal tissue also showed traces of a methylated RARβ gene in the cell population. Our hypothesis is that methylation of RARβ in normal colon tissue is possibly an early preneoplastic event that favors the development of malignancy. In accord with our hypothesis is the report that human breast tumors and normal adjacent tissue often do not express RARβ mRNA; however, no data on DNA methylation was presented.[44] It is of interest to note that in normal human colon tissue methylation of another gene, the estrogen receptor, was observed to increase with age and may be an early event that predisposes to colon tumorigenesis.[45]

Microsatellite analysis, performed as described previously,[46] indicated that one (no. 2) of the six colon tumor samples with hypermethylation of the RARβ gene showed microsatellite instability; only two of eight tumor samples without methylation of RARβ showed microsatellite instability (D Sinnett, unpublished data). It has been reported that there is a good correlation with microsatellite instability and promoter hypermethylation for malignancy-related genes in colon cancer.[13]

The role of RARβ in the development of colon cancer is not known. $RAR\beta_2$ has been proposed to act as a tumor suppressor gene.[24,34] It is possible that the expression of RARβ may favor the differentiation of colon epithelium decreasing the incidence of cellular replication resulting in a reduction in genetic or epigenetic changes that can lead to malignancy. The neoplastic progression may occur for lung cancer. It is interesting to note that one patient with stage IV non-small cell lung cancer survived more than 6 years after 5-Aza-CdR treatment.[47] It is possible that the activation of RARβ in the lung tumor of this patient by the inhibitor of DNA methylation may have contributed to the long survival time. Future investigations will determine if the methylation status of the RARβ gene is an important prognostic or diagnostic marker for colon and lung cancer. The MSP assay for methylation of RARβ may be a useful tool for these studies and to monitor if 5-Aza-CdR therapy can demethylate the gene.

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Addendum

The detailed sequence data of the promoter region of RARβ will be published in the PhD thesis (Université de Montréal) of SC entitled: 'Récepteur de l'acide rétinoïque bêta: déméthylation et activation par la 5-Aza-2'-désoxycytidine dans le cancer'.

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