

Retinoid X Receptor- α Expression Suppression and its Promoter Methylation Associates with β -catenin Expression in Colorectal Cancer

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ABSTRACT

AIM: Whether retinoid X receptor- α (RXR α) expression suppression is related to epigenetic regulation in colorectal cancer (CRC) is unclear. Our current study is to investigate the promoter methylation status of RXR α and correlated the results with β -catenin expression and clinicopathological characteristics in CRC.

METHODS: Real-time PCR, Western blot, immunohistochemistry staining, methylation special PCR, and methylation sensitive-high resolution melting (MS-HRM) curve analysis were used to detect the expression of RXR α and β -catenin and RXR α promoter methylation status in CRC cells and tissues.

RESULTS: Our study showed that RXR α low expression rate was significantly higher in 202 samples of CRC tissues than that in adjacent non-tumor colorectal mucosa (ANM) tissues ($P < 0.001$). CRC patients had a significantly shorter overall survival rate in the low RXR α expression group compared with that in the high RXR α expression group ($p = 0.038$). There was a significant correlation between RXR α low expression and β -catenin abnormal expression in CRC tissues. The promoter methylation of RXR α was detected in HT29, HCT116, SW480, SW620, and LoVo except SW116 and NCM460, all 8 pairs of fresh CRC and ANM tissues, all 13 samples of paraffin-embedded ANM, 21 CRC, and 8 lymph node metastatic CRC tissues by methylation special PCR. Further MS-HRM curve

analysis showed that the promoter methylation of RXR α was detected in 76.92% (10/13) of ANM, 85.71% (18/21) of CRC, and 75.0% (6/8) of lymph node metastatic CRC tissues, respectively. The mean methylation degree of RXR α was significantly higher in CRC and lymph node metastatic CRC than that in ANM tissues, respectively ($P = 0.038$, $P = 0.035$). Methylation degree of RXR α was decreased in SW620 and LoVo cells treated with 5-Aza-2'-Deoxycytidine. Furthermore, RXR α and β -catenin mRNA and protein expression dramatically elevated and suppressed in LoVo cells treated with 5-Aza-2'-Deoxycytidine at different concentrations and times compared with the control group, respectively.

CONCLUSION: Our results first demonstrate that RXR α low expression associates with poor prognosis of CRC patients. The promoter methylation of RXR α might be related to RXR α expression suppression in CRC. Targeting RXR α promoter methylation might be a novel therapeutic strategy for CRC patients.

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Key words: Retinoid X receptor- α ; Promoter methylation; β -catenin; Colorectal cancer

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies worldwide. The retinoid X receptors (RXRs) are nuclear receptors and are members of the superfamily of ligand-inducible transcriptional regulatory factors that mediates the anti-cancer function of retinoids (natural retinoic acids and their synthetic derivatives). Retinoid X receptors have three different isotypes- α , β and γ . RXR could heterodimerise with retinoid acid receptor (RAR) and other receptors such as thyroid hormone receptors, vitamin

D receptors and peroxisome-proliferator-activated receptors^[1-3]. RXR expression associates with a variety of malignant tumors and prognosis of patients with these tumors^[3-9].

The Wnt/ β -catenin signaling pathway is tightly regulated and has important functions in development, tissue homeostasis, and regeneration. Oncogenic activation of the Wnt/ β -catenin signaling pathway by mutation in adenomatous polyposis coli or β -catenin, which results in the cytoplasmic and nuclear β -catenin accumulation and in β -catenin/T-cell factor (TCF) 4-regulated transcription of TCF target genes such as cyclin D1 and c-Myc, is mandatory for the initial neoplastic transformation of intestinal epithelium^[10,11].

Recently, instances of crosstalk between retinoid signaling pathways and the Wnt/ β -catenin signaling have been reported^[12,13]. Our previous study showed that RXR α directly interacted with β -catenin and that RXR α directly inhibited β -catenin transcriptional activity and protein expression in CRC cell lines^[14]. Further study showed that suppression of RXR α and aberrant β -catenin expression significantly associated with progression of CRC^[8]. The mechanism underlying the impaired expression of RXR α in CRC remains unclear. Aberrant methylation of promoter CpG islands is known to be a major mechanism for inactivation of tumor suppressor genes^[15]. Lee *et al.*^[9] have reported that methylation of RXR α , RXR β , and RXR γ is 5.7%, 4.3%, and 23.7% in lung cancer, respectively. In this study, we first investigated the promoter methylation status of RXR α and correlated the results with β -catenin expression and clinicopathological characteristics in large series of CRC.

MATERIALS AND METHODS

Cell lines and cell culture

Human CRC cell lines LoVo and SW116 were maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA). HT29 was cultured in Dulbecco's modified Eagle's medium (Invitrogen). SW480 and SW620 were grown in Leibovitz's L-15 Medium (Invitrogen). HCT116 was maintained in McCoy's 5A Medium (Invitrogen). The human colonic epithelial cell line NCM460 was cultured in RPMI-1640 medium. All medium was supplemented with 10% (v/v) fetal bovine serum (GIBCO, Carlsbad, CA), 1 \times antibiotic/antimycotic (100 units/mL streptomycin, 100 units/mL penicillin, and 0.25 mg/mL amphotericin B). All cell lines were cultured in humidified incubator at 37°C with 5% CO₂. Inhibitor of DNA methyltransferase-5-Aza-2'-Deoxycytidine (Sigma, St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) and diluted in a series concentration.

Patient information and tissue specimens

Eight pairs of fresh CRC tissues and adjacent non-tumor colorectal mucosa (ANM) tissues, 202 pairs of paraffin-embedded, archived CRC and ANM tissues and 75 samples of lymph node metastatic CRC tissues between 2002 and 2008 were collected from Department of Pathology, the first Affiliated Hospital, Sun Yat-sen University, Guangzhou, China. No patients had received chemotherapy or radiotherapy before surgery. The histopathology of the disease was determined by two pathologists according to the criteria of the World Health Organization. Clinical staging was done according to the Dukes' classification. For the research purposes of these clinical materials, prior patient's consents and approval from the Institutional Research Ethics Committee were obtained. Follow-up information was available for all patients. Detailed clinical information about these patients including sex, age, histological differentiation, Dukes' classification, TNM stage, T classification, N classification, and distant metastasis status, is summarized in table 1.

RNA extraction and Real-time polymerase chain reaction (PCR)

Total RNA from CRC cell lines, fresh CRC tissues, and ANM tissues was extracted using the Trizol reagent (Invitrogen) according to the manufacturer's instruction. Real-time PCR was done according to the manufacturer's protocol from TIANGEN BIOTECH (Beijing, China) CO., LTD. The primer sequences used for RXR α were followed: forward: 5' GCA AGC TGG TGT GTC ATC AGC AAA; reverse: 5' ACA GAG GGC AGC TCA TGT TCT CAT. The primer sequences used for β -catenin were followed: forward: 5' TTG AAA ATC CAG CGT GGA CA; reverse: 5' TCG AGT CAT TGC ATA CTG TC. The geometric mean of housekeeping gene GAPDH was used to normalize the variability at expression levels.

DNA extraction and methylation special PCR

DNA was extracted from CRC cell lines and tissues using DNA isolation kits (Qiagen, Hilden, Germany). To investigate the methylation status of RXR α , methylation special PCR with primers specific for the methylated and unmethylated alleles of each gene after treatment of the genomic DNA with sodium bisulfite using EpiTect Fast Bisulfite Conversion Kits (Qiagen) was performed. Two CpG islands were found in the promoter sequence of RXR α , Island 1, size: 352 bp (622-973); Island 2, size: 894 bp (1053-1946) (<http://www.urogene.org/>), thus enabling us to design methylation special PCR primer around the CpG island site with MethPrimer computer program (<http://www.urogene.org/methprimer/> and Methyl Primer

Table 1 RXR α and β -catenin expression and their association with clinicopathological features of CRC.

Variable		Total No.	RXR α expression				β -catenin expression			
			Low	High	χ^2	p value	Normal	Abnormal	χ^2	p value
Gender	Male	118	73	45	0.142	0.706	12	106	0.160	0.90
	Female	84	55	29			9	75		
Age(years)	≤ 62.5	102	62	40	0.592	0.442	13	89	0.122	0.269
	> 62.5	100	66	34			8	92		
Histological differentiation	Well	38	28	10	6.098	0.047	10	28	12.813	0.002
	Moderately	112	74	38			7	105		
	Poorly	52	26	26			4	48		
Duke's stage	A	20	14	6	13.973	0.003	6	14	11.677	0.009
	B	83	54	29			9	74		
	C	75	38	37			3	72		
	D	24	22	2			3	21		
T classification	T1-T2	103	68	35	0.637	0.425	15	88	3.918	0.048
	T3-T4	99	60	39			6	93		
N classification	N0	127	90	37	8.288	0.004	18	109	5.293	0.022
	N1-N2	75	38	37			3	72		
M classification	M0	178	106	72	9.937	0.002	18	160	0.129	0.719
	M1	24	22	2			3	21		

Express v1.0). The methylated primer sequences for RXR α were followed: forward: 5' GAT TTT TTA TCG GAA TAG GTT ATC G; reverse: 5' GCG TAT AAA CAA CCA TAA CAC GAC. The unmethylated primer sequences for RXR α were followed: forward: 5' TGT TTT TTG TTG TTG GGG TAT ATT; reverse: 5' CCA TTA TCT ACA CAA CCA CAC A.

Methylation sensitive-high resolution melting (MS-HRM) curve analysis

The designed sequences for RXR α primers were: forward 5'-GGG TAT TTT TGT TTT AAG TGG AAT-3', Reverse: 5'-CAT AAC ACG ACC CAA AAA TAA CA-3'. MS-HRM was carried out on Light Cycler480 (Roche). PCR was performed in 20 μ L volume containing 2 μ L 10 \times PCR Buffer, 25 mM MgCl₂, 0.5 μ L 10 mM dNTPs, 10 μ M of each primer, 1 μ L 20 \times Eva-Green dye, 1U FastStart Taq DNA Polymerase, 1 μ L of bisulfite modified DNA template, 12.3 μ L purified water. The cycling condition used was as follows: the initial denaturation at 95 $^{\circ}$ C for 5 minutes, followed by 50 cycles of denaturation at 95 $^{\circ}$ C for 30 seconds, primer annealing at 60 $^{\circ}$ C for 15 seconds, and the extension step at 72 $^{\circ}$ C for 25 seconds, followed by an HRM step at 95 $^{\circ}$ C for 1 minute, 40 $^{\circ}$ C for 1 minute, 65 $^{\circ}$ C for 1 second and continuous acquisition to 95 $^{\circ}$ C at 25 acquisitions per 1 $^{\circ}$ C, followed by the final step 40 $^{\circ}$ C for 30 seconds. Methylated and unmethylated genomic templates were used to calibrate the quantitative measurements of methylation. Bisulfite converted human control DNA including methylated and unmethylated (Qiagen) was used as 100% methylated control DNA and unmethylated control DNA, respectively. Methylation standards were constructed by diluting 100% methylated control DNA in a pool of unmethylated control DNA at levels of 100%, 75%, 50%, 25%, 5%, and 0%. These standards were included in each experimental run. Each reaction was performed in duplicate.

Western blot analysis

As described previously^[8], RXR α antibody and β -catenin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) were used to probe the alteration of protein at 4 $^{\circ}$ C for 12 hours. Signal was detected by enhanced chemoluminescence techniques (Amersham Life Science, Piscataway, NJ). GAPDH (Sigma, St. Louis, MO) was used as loading control.

Immunohistochemistry

The sections were deparaffinized, rehydrated in serially graded ethanol, and heated in citric buffer (pH 6.0) once for 5 minutes in a microwave oven for antigen retrieval. They were then washed with distilled water, blocked with 3% hydrogen peroxide and incubated with the primary antibodies including RXR α (dilution 1:200) and β -catenin (dilution 1:200, Santa Cruz Biotechnology) at 4 $^{\circ}$ C for 12 hours. After washing with a 0.01 mol/L concentration of PBS, the sections were incubated with EnVision-HRP secondary antibody (Dako, Carpinteria, CA) for 30 minutes at room temperature, washed with a 0.01 mol/L concentration of PBS, stained with 0.5% diaminobenzidine and counterstained with Mayer's haematoxylin, then air dried, and mounted with glycerol gelatin.

Evaluation of immunohistochemistry staining

Immunohistochemistry staining was independently assessed by two researchers who were unaware of the specific proteins being assessed or their putative role in CRC. The degree of RXR α immunostaining was based on both the proportion of positively stained tumor cells and intensity of staining. The proportion of positively stained tumor

cells was scored as follows: 0 (no positive tumor cells), 1 (<10% positive tumor cells), 2 (10-50% positive tumor cells), and 3 (>50% positive tumor cells). Staining intensity was classified according to the following criteria: 0 (no staining); 1 (weak staining=light yellow), 2 (moderate staining=yellow brown), and 3 (strong staining=brown). Staining index was calculated as the staining intensity score \times the proportion score. Using this method, we evaluated the expression of RXR α in colorectal specimen by determining the staining index, with scores 0, 1, 2, 3, 4, 6, or 9. The staining index score of 4 (a cutoff point) was used to distinguish between low and high expression of RXR α . The staining of β -catenin was scored according to Maruyama's method^[16]. When more than 70% of carcinoma cells were positively stained for membranous β -catenin, the cells was classified as β -catenin normal expression; if more than 10% of carcinoma cells were positively stained for cytoplasm or nuclei was regarded as β -catenin abnormal expression.

Statistical analysis

Chi-square test and Fisher exact test were used to compare the levels of RXR α and β -catenin expression with both different groups and various clinicopathological parameters. Overall survival (OS) time of CRC patients was analyzed by Kaplan-Meier curve and log-rank test. The correlation of β -catenin and RXR α expression was analyzed by Spearman's correlation coefficients. All statistical analyses were performed using SPSS 16.0 statistics software. $P < 0.05$ was set to be statistically significant.

RESULTS

RXR α and β -catenin mRNA and protein expression in CRC cell lines and fresh tissues

As shown in Figure 1, RXR α mRNA expression was higher in CRC cell lines including SW116, HT29, HCT116, SW480, SW620, and LoVo compared with the human colonic epithelial cell line NCM460 by real-time PCR analysis. However, RXR α protein expression was lower in NCM460, SW620, and LoVo cells than that in other CRC cell lines including SW116, HT29, HCT116, and SW480 by western blot analysis. Furthermore, RXR α protein expression was dramatically lower in LoVo and SW620 than NCM460 cells. The mRNA and protein expression of β -catenin were higher in such 6 CRC cell lines compared with NCM460 cells by real-time PCR and western blot analysis, respectively. In addition, RXR α mRNA expression was dramatically lower in 8 samples of fresh CRC tissues than that in their corresponding ANM tissues except one sample (case 4) by real-time PCR analysis (Figure 1D).

Relationship between expression of RXR α and β -catenin and clinicopathological features of CRC

Of 202 samples of CRC tissues, 128 samples (63.4%) were RXR α low expression, 74 samples (36.6%) were RXR α high expression. However, of 131 samples of ANM tissues, only 53 samples (40.5%) were RXR α low expression, 78 samples (59.5%) were RXR α high expression. RXR α low expression rate was significantly higher in CRC tissues than that in ANM tissues ($P < 0.001$, figure 2A-B). Furthermore, RXR α low expression was significantly associated with histological differentiation ($P = 0.047$), Dukes' classification ($P = 0.003$), N classification ($P = 0.004$), and M classification ($P = 0.002$). However, no significant association of RXR α expression with gender, age, and T classification ($P > 0.05$) was found. Kaplan-Meier analysis showed that CRC patients had a significantly lower OS rate in RXR α low expression group compared with that in RXR α high

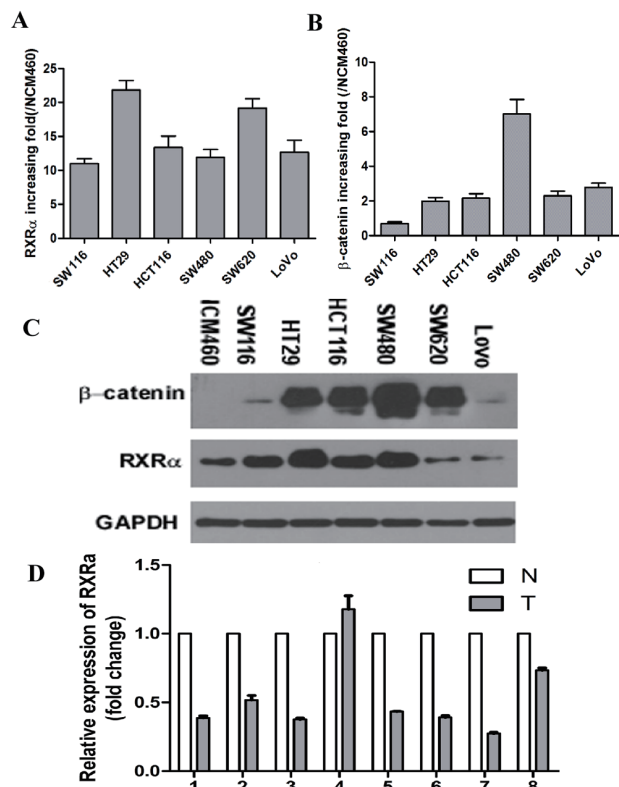


Figure 1 A-B: RXR α and β -catenin mRNA expression in CRC cell lines compared with human colonic epithelial cell line NCM460 by real-time PCR analysis; C RXR α and β -catenin protein expression in CRC cell lines and NCM460 by western blot analysis; D RXR α mRNA expression in 8 pairs of fresh CRC tissues and their corresponding adjacent non-tumor colorectal mucosa (ANM) tissues by real-time PCR analysis.

expression group ($p=0.038$, figure 2C). The OS time for patients with RXR α low expression was 57.19 ± 3.93 months (range: 49.49-64.90 months), which was significantly shorter than 67.19 ± 2.73 months (range: 61.84-72.54 months) of OS time for patients with RXR α high expression, respectively. The OS rate for patients with RXR α low expression at 3 years and 5 years was 69.86% and 67.12%, respectively, compared with 79.53% and 74.16% for patients with RXR α high expression at 3 years and 5 years, respectively. 181 samples (89.6%, 181/202) were β -catenin abnormal expression, 21 samples (36.6%, 21/202) were β -catenin normal expression. However, of 115 samples of ANM tissues, only 8 samples (7.0%) were β -catenin abnormal expression, 107 samples (93.0%) were β -catenin normal expression. β -catenin abnormal expression was significantly higher in CRC tissues than that in ANM tissues ($P<0.001$, figure 2D). Furthermore, β -catenin abnormal expression was significantly associated with poor histological differentiation ($P=0.002$), high Dukes' classification ($P=0.009$), T classification ($P=0.048$), and N classification ($P=0.022$). However, no significant association of β -catenin abnormal expression with gender, age, and M classification ($P>0.05$) was found (Table 1).

Of 202 samples of CRC tissues, 110 samples were β -catenin abnormal expression in RXR α low expression group, 71 samples were β -catenin abnormal expression in RXR α high expression group. There was a significant correlation between RXR α low expression and β -catenin abnormal expression in CRC tissues ($P=0.028$, table 2).

Promoter methylation status of RXR α in CRC

The promoter methylation status of RXR α was first determined in 6 CRC cell lines and normal colonic mucosa cell line NCM460

Table 2 The association between RXR α and β -catenin expression in CRC tissue.

Variable	RXR α expression		P value	
	Low (%)	High (%)		
β -catenin expression	Normal	18 (8.9)	3 (1.5)	0.025
	Abnormal	110 (54.5)	71 (35.1)	

by methylation special PCR analysis. As shown in figure 3A, unmethylated bands were detected in all cell lines. Methylated bands were detected in HT29, HCT116, SW480, SW620, and LoVo cells except SW116 and NCM460. Moreover, methylated bands were stronger in SW620 and LoVo cells than those in other CRC cell lines. In addition, methylated bands were found in 8 pairs of fresh CRC and ANM tissues (Figure 3B). Further study showed that methylated bands were found in all 13 samples (100%) of paraffin-embedded ANM tissues, 21 samples (100%) of CRC tissues, and 8 samples (100%) of lymph node metastatic CRC tissues.

To confirm the above results, MS-HRM analysis was performed to detect methylation status and methylation degree in such 13 samples of paraffin-embedded ANM tissues, 21 samples of CRC tissues, and 8 samples of lymph node metastatic CRC tissues. As shown in figure 3C and table 3, the promoter methylation of RXR α was detected in 10 samples (76.92%, 10/13) of ANM, 18 samples (85.71%, 18/21) of CRC, and 6 samples (75.0%, 6/8) of lymph node metastatic CRC tissues, respectively. Furthermore, the mean methylation degree was 5% (95% confidence interval: -0.57-10.57) in ANM, 16.57% (95% confidence interval: 6.81-26.33) in CRC, and 26.13% (95% confidence interval: -0.54-52.79) in lymph node metastatic CRC tissues. The mean methylation degree of RXR α was significantly higher in CRC and lymph node metastatic CRC tissues than that in ANM tissues, respectively ($P=0.038$ and $P=0.035$, figure 3D). Of 18 samples of CRC with promoter methylation of RXR α , 12 samples (66.6%) were RXR α low expression by immunohistochemistry staining. There was no significant association between promoter methylation of RXR α and clinicopathological features of CRC ($P>0.05$).

Table 3 The promoter methylation of RXR α in CRC using methylation sensitive-high resolution melting curve analysis.

Variable	Case No.	Methylation No. (%)	Mean methylation degree (%)	95% confidence interval
Adjacent non-tumor colorectal mucosa	13	10 (76.92)	5.0	-0.57-10.57
CRC	21	18 (85.71)	16.57	6.81-26.33
Lymph node metastatic CRC	8	6 (75.0)	26.13	-0.54-52.79

Furthermore, MS-HRM analysis was performed to detect methylation status and methylation degree in NCM460 and CRC cell lines. The methylation degree of RXR α was 0%, 0%, 25-50%, 5-25%, 25-50%, 50-75%, and 50% in NCM460, SW116, HT29, HCT116, SW480, SW620, and LoVo cells, respectively. The results showed that methylation degree of RXR α was higher in SW620 and LoVo cells than that in other CRC cells and NCM460. Then, methylation degree of RXR α was decreased in SW620 and LoVo cells treated with inhibitor of DNA methyltransferase -5-Aza-2'-Deoxycytidine at 1 μ M, 5 μ M, and 10 μ M for 1 day and 3 days compared with the control group (Table 4).

As shown in figure 4, RXR α mRNA and protein expression dramatically elevated in LoVo cells treated with inhibitor of DNA methyltransferase -5-Aza-2'-Deoxycytidine at 1 μ M, 5 μ M, and 10 μ M for 1 day and 3 days compared with the control group by real-time PCR and western blot analysis, respectively. However, β -catenin mRNA and protein expression dramatically suppressed in LoVo cells

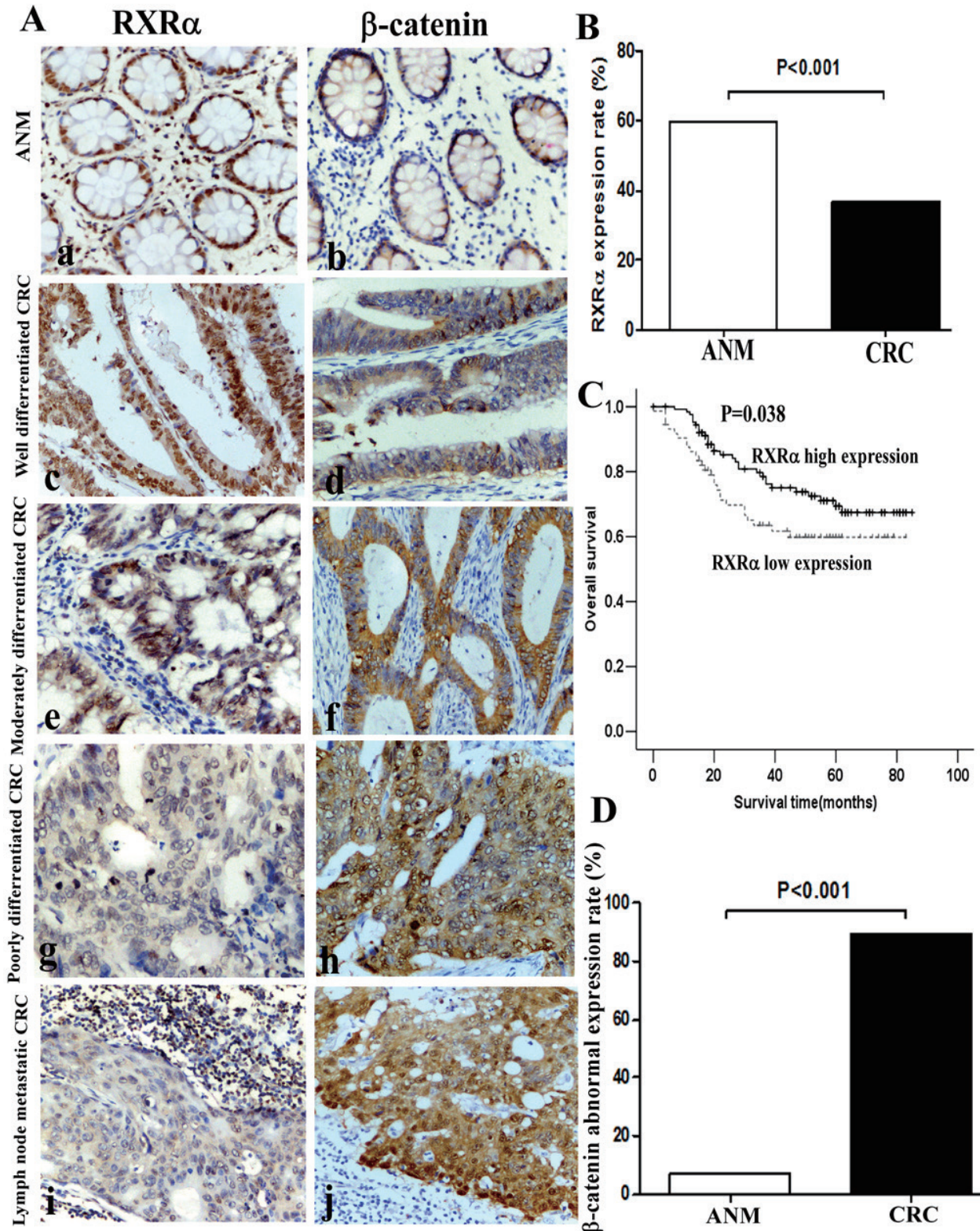


Figure 2 A: RXR α and β -catenin protein expression in ANM (a, b), well differentiated CRC (c, d), moderately differentiated CRC (e, f), poorly differentiated CRC (g, h), and lymph node metastatic CRC tissues (i, j) by immunohistochemistry staining, $\times 400$; B: RXR α expression was significantly suppressed in CRC compared with ANM ($P < 0.001$); C: Overall survival of CRC patients with different levels of RXR α expression by Kaplan-Meier analysis; the log-rank test was used to compare the survival curves between the two groups; D: β -catenin abnormal expression was elevated in CRC compared with ANM ($P < 0.001$).

Table 4 The promoter methylation of RXR α in CRC cell lines treated with 5-Aza-2'-Deoxycytidine using methylation sensitive-high resolution melting curve analysis.

Variable	1 day with 5-Aza-2'-Deoxycytidine			3 days with 5-Aza-2'-Deoxycytidine			Control (3 days)
	1 μ m	5 μ m	10 μ m	1 μ m	5 μ m	10 μ m	0 μ m
Methylation degree in SW620	50%	50%	50%	25-50%	50%	50%	50-75%
Methylation degree in LoVo	25-50%	25-50%	0%	25%	25%	25%	50%

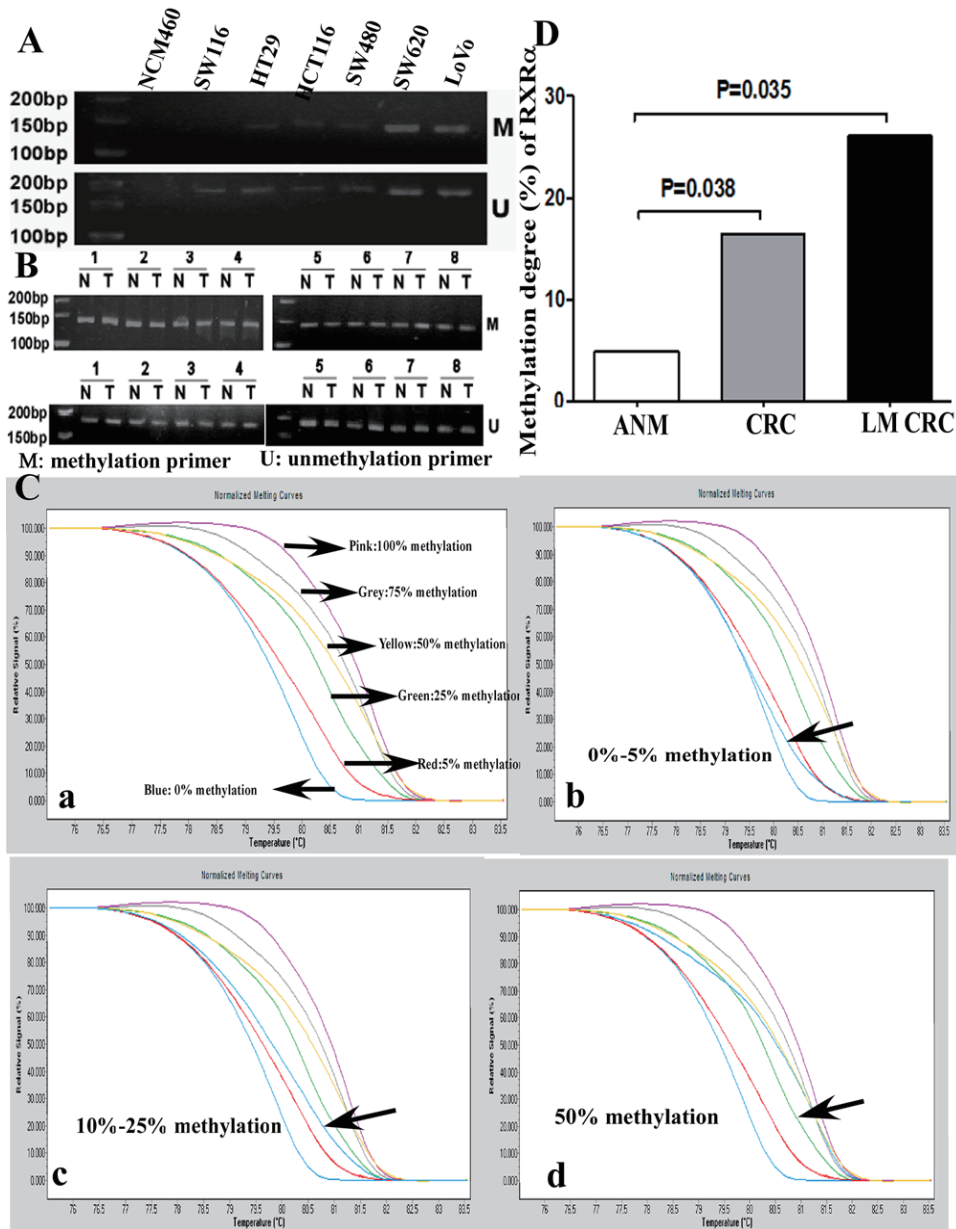


Figure 3 A-B: The promoter methylation status of RXR α in CRC cell lines and NCM460 cells (A), and 8 pairs of fresh CRC and ANM tissues (B) using methylation special PCR analysis; C: The promoter methylation status of RXR α in standard methylation sample (a), ANM (b), CRC(c), and lymph node metastatic CRC (d) tissues using methylation sensitive-high resolution melting curve analysis; D: The mean methylation degree of RXR α was significantly higher in CRC tissues and lymph node metastatic CRC (LM CRC) tissues than that in ANM tissues, respectively ($P=0.038$ and $P=0.035$).

treated with 5-Aza-2'-Deoxycytidine at 1 μ M, 5 μ M, and 10 μ M for 1 day and 3 days compared with the control group by real-time PCR and western blot analysis, respectively.

DISCUSSION

RXR α s are nuclear receptors for retinoids that play a critical role in the regulation of growth and differentiation in normal and tumor cells^[3,5-9,17]. Our previous study showed that RXR α directly inhibited β -catenin transcriptional activity and protein expression in CRC cell lines^[14]. Further study showed that suppression of RXR α and aberrant β -catenin expression significantly associated with progression of CRC^[8]. Our current results showed that RXR α protein expression was lower in NCM460, SW620, and LoVo cells than that in SW116, HT29, HCT116, and SW480 by western blot analysis. Furthermore, RXR α protein expression was dramatically lower in LoVo and

SW620 than NCM460 cells. Further study showed that RXR α low expression rate was significantly higher in 202 samples of CRC tissues than that in ANM tissues. β -catenin abnormal expression was significantly higher in CRC tissues than that in ANM tissues. There was a significant association between RXR α low expression and β -catenin abnormal expression in CRC tissues. To our knowledge, our results first showed that CRC patients had a significantly lower OS rate in RXR α low expression group compared with that in RXR α high expression group.

Lee *et al*^[9] have reported promoter methylation of RXR α is 5.7% in lung cancer. To further investigate the mechanism underlying the impaired expression of RXR α in CRC, we detected methylation status of RXR α in CRC cell lines and tissues using methylation specific PCR analysis. Our result showed that promoter methylation of RXR α was detected in HT29, HCT116, SW480, SW620, and LoVo except SW116 and NCM460 cells. Moreover, methylated

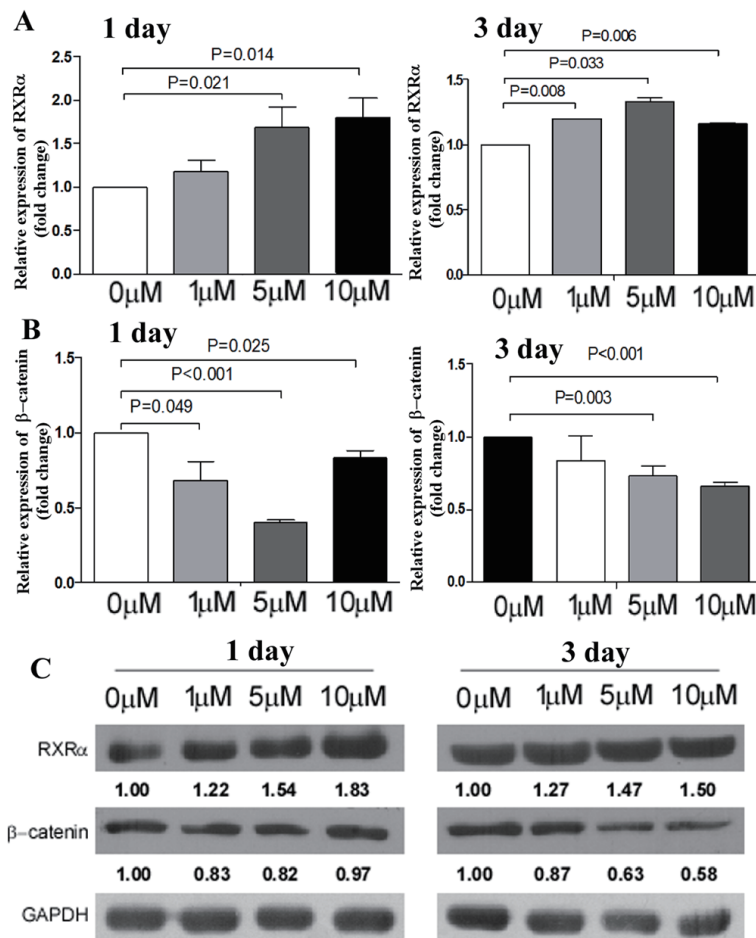


Figure 4 RXR α and β -catenin mRNA (A-B) and protein (C) expression dramatically elevated and suppressed in LoVo cells treated with 5-Aza-2'-Deoxycytidine at 1 μ M, 5 μ M, and 10 μ M for 1 day and 3 days compared with the control group by real-time PCR and western blot analysis. The relative quantification of bands in western blot was a ratio neutralized to GAPDH.

bands were stronger in SW620 and LoVo cells than those in other CRC cell lines. Moreover, methylation degree of RXR α was higher in SW620 and LoVo cells than that in other CRC cells and NCM460 by MS-HRM analysis. Further study showed that methylation degree of RXR α was decreased in SW620 and LoVo cells treated with inhibitor of DNA methyltransferase 5-Aza-2'-Deoxycytidine. The result was consistent with the findings that RXR α protein expression was dramatically lower in SW620 and LoVo than NCM460 and other CRC cells. Consistent with our previous report^[8], methylation of RXR α was found in 8 pairs of fresh CRC and ANM tissues. Further study showed that methylation of RXR α was found in all 13 samples of paraffin-embedded ANM tissues, 21 samples of CRC tissues, and 8 samples of lymph node metastatic CRC tissues. To confirm the above results, MS-HRM analysis showed that promoter methylation of RXR α was detected in 10 samples (76.92%, 10/13) of ANM, 18 samples (85.71%, 18/21) of CRC, and 6 samples (75.0%, 6/8) of lymph node metastatic CRC tissues, respectively. Furthermore, the mean methylation degree of RXR α was significantly higher in CRC and lymph node metastatic CRC tissues than that in ANM tissues, respectively. The result was consistent with the findings that RXR α protein expression was lower in CRC and lymph node metastatic CRC tissues than that in ANM tissues by immunohistochemistry staining. In addition, this finding suggests that MS-HRM analysis is much better than methylation specific PCR for detecting methylation status of RXR α in CRC. MS-HRM analysis is much stable, reliable, and sensitive compared with methylation specific PCR^[18]. Most

importantly, MS-HRM analysis could provide not only methylation status but also methylation degree for CRC samples. Pande *et al*^[5] have reported single nucleotide polymorphisms in RXR α may contribute to breast cancer disease-free survival (DFS) and predict for poorer or improved DFS in patients according to type of systemic treatment received. Lee *et al*^[9] have reported RXR γ methylation is associated with a significantly worse survival in lung cancer patients with never-smokers. However, no significant association between promoter methylation of RXR α and clinicopathological features of CRC was found in our series. The reason might be related to the limited CRC samples analyzed by MS-HRM analysis. This issue needs further study in large samples of CRC.

To further investigate whether RXR α and β -catenin expression altered in CRC cells treated with inhibitor of DNA methyltransferase, our results showed that RXR α and β -catenin mRNA and protein expression dramatically elevated and suppressed in LoVo cells treated with 5-Aza-2'-Deoxycytidine at different concentrations and times compared with the control group, respectively. Volate *et al*^[19] have reported a low concentration of green tea significantly decreased CpG methylation of RXR α , increased RXR α expression, and inhibit intestinal tumorigenesis in the Apc(Min/+) mouse. Xiao *et al*^[20] have reported green tea polyphenols inhibit colorectal aberrant crypt foci formation and prevent oncogenic changes in dysplastic ACF in azoxymethane-treated F344 rats partially by preventing the loss of RXR α expression. Targeting truncated RXR α by a natural product molecule (CF31) induces TNF α -dependent apoptosis in various

cancer cell lines^[21]. Whether inhibitor of DNA methyltransferase treatment has an effect on cell biological functions in CRC cells with RXR α promoter methylation needs further study.

In summary, our results first demonstrate that RXR α low expression associates with poor prognosis of CRC patients. The promoter methylation of RXR α might be related to RXR α expression suppression in CRC. Our data suggest that targeting RXR α promoter methylation might be a novel therapeutic strategy for CRC patients.

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CONFLICT OF INTERESTS

There are no conflicts of interest with regard to the present study.

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