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# Suppression of retinoid X receptor alpha and aberrant β-catenin expression significantly associates with progression of colorectal carcinoma

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ABSTRACT

To investigate retinoid X receptor alpha (RXRα) and β-catenin expression and their relationship with the clinicopathological features of colorectal carcinoma (CRC). Real-time PCR and western blot analyses revealed that β-catenin and RXRα expression at both mRNA and protein levels in four pairs of fresh CRC and adjacent non-tumour tissues (ANT) dramatically was increased and decreased in CRC compared with ANT, respectively. Furthermore, RXRα expression at both mRNA and protein levels was downregulated in higher histological grade CRC. Immunohistochemistry staining in 120 cases of CRC and 60 cases of lymph node metastatic carcinoma of CRC showed that RXRα expression was significantly suppressed in CRC compared with ANT ($P < 0.001$) and low expression of RXRα in CRC was significantly associated with histological grade ($P < 0.001$), TNM stage ($P = 0.022$) and N classification ($P = 0.002$). The aberrant (accumulated cytoplasm or/and nuclei) expression of β-catenin was higher in CRC than that in ANT ($P < 0.001$) and associated with histological grade ($P = 0.001$) and N classification ($P = 0.002$). Moreover, there was a close relationship between low RXRα expression and aberrant β-catenin expression in CRC ($P = 0.032$). Taken together with our previous study, aberrant β-catenin expression upregulated by suppression of RXRα may play a crucial role in pathogenesis and progression of CRC.



## 1. Introduction

Colorectal carcinoma (CRC) is one of the most common malignancies worldwide. Oncogenic activation of the Wnt-signalling pathway by mutations in adenomatous polyposis coli (APC) or β-catenin, which results in the accumulation and nuclear translocation of β-catenin and in β-catenin/T-cell factor (TCF) 4-regulated transcription of TCF target genes such as cyclin D1, c-Myc and Phopholipase D, is mandatory for the initial neoplastic transformation of intestinal epithelium.[1–3] Targeting the Wnt signalling pathway may be a fruitful strategy for targeting chemotherapy-resistant colon cancer.[4] 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).[5–7] RXRs have three isotypes—α, β and γ. RXRs could heterodimerise with retinoid acid receptor (RAR) and other receptors such as thyroid hormone receptors, vitamin D receptors and peroxisome-proliferator-activated receptors

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(PPARs).[8,9] In addition to forming heterodimers, RXRs bind to 9-cis-retinoid acid and form functionally active homodimers that bind to the target DNA sequences (RXRE) and activate gene transcription.[10–12]

Recently, instances of crosstalk between retinoid signalling pathways and the Wnt/β-catenin signalling have been reported. It has been reported that RARα interacts with β-catenin and inhibits β-catenin-mediated gene transcription.[13–15] Our previous study showed that RXRα directly interacted with β-catenin, and that RXRα directly inhibited β-catenin transcriptional activity and protein expression in colorectal cancer cell lines. However, β-catenin transcriptional activity and protein expression were increased when RXRα was knockdown by targeting RXRα small RNA interfering. But, overexpression or reduction of β-catenin did not affect RXRα expression.[16] No studies to date have evaluated the association of RXRα and β-catenin expression with the clinicopathological features of CRC. The purpose of this study was, therefore, to comprehensively evaluate the relationship between the expression of RXRα and β-catenin and the clinicopathological features of CRC.

## 2. Materials and methods

### 2.1. Patient information and tissue specimens

Four pairs of fresh CRC tissues and adjacent non-tumour tissues (ANT), and a total of 120 cases of paraffin-embedded, archived CRC tissues, ANT and 60 cases of lymph node metastatic carcinoma tissues between 2008 and 2010 were collected from Department of Pathology, the first Affiliated Hospital, Sun Yat-Sen University. 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 Organisation. Clinical staging was done according to the current International Union against Cancer (UICC) Classification. For the research purposes of these clinical materials, prior patient's consents and approval from the Institutional Research Ethics Committee were obtained. Detailed clinical information about these patients, including age, gender, clinical stage, T classification, N classification and distant metastasis status, was summarised in Table 1.

### 2.2. Real-time PCR analysis

Total RNA from primary tumour samples and ANT was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Real-Time PCR was done according to the manufacturer's protocol from TIANGEN BIOTECH(BEIJING) CO., Ltd. 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. Primers for RXRα were followed: forward: 5′ GCA AGC TGG TGT GTC ATC AGC AAA; reverse: 5′ ACA GAG GGC AGC TCA TGT TCT CAT. The geometric mean of housekeeping gene GAPDH was used to normalise the variability at expression levels.

### 2.3. Western blot analysis

Four pairs of fresh surgical specimens from CRC and ANT were promptly removed and epithelial cells from ANT were

**Table 1 – β-Catenin and RXRα expression in CRC and their association with clinicopathological features of CRC.**

| Variables | No. patients | β-Catenin | | | | RXRα | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Normal | Ectopic | $\chi^2$ | P value | Low | High | $\chi^2$ | P value |
| *Sex* | | | | | | | | | |
| Male | 68 | 27 | 41 | 1.266 | 0.260 | 40 | 28 | 0.112 | 0.737 |
| Female | 52 | 26 | 26 | | | 29 | 23 | | |
| *Age* | | | | | | | | | |
| ⩽60 | 58 | 25 | 33 | 0.051 | 0.821 | 37 | 21 | 1.819 | 0.177 |
| >60 | 62 | 28 | 34 | | | 32 | 30 | | |
| *Histological differentiation* | | | | | | | | | |
| Well | 5 | 4 | 1 | 12.293 | 0.001 | 0 | 5 | 18.052 | <0.001 |
| Moderately | 58 | 33 | 25 | | | 26 | 32 | | |
| Poorly | 57 | 16 | 41 | | | 43 | 14 | | |
| *UICC stage* | | | | | | | | | |
| I ∼ II | 56 | 21 | 35 | 1.892 | 0.169 | 26 | 30 | 5.267 | 0.022 |
| III ∼ IV | 64 | 32 | 32 | | | 43 | 21 | | |
| *T classification* | | | | | | | | | |
| T1 ∼ T2 | 78 | 34 | 44 | 0.030 | 0.862 | 45 | 33 | 0.003 | 0.954 |
| T3 ∼ T4 | 42 | 19 | 23 | | | 24 | 18 | | |
| *N classification* | | | | | | | | | |
| N0 | 60 | 35 | 25 | 9.766 | 0.002 | 17 | 43 | 9.855 | 0.002 |
| N1 ∼ N2 | 60 | 18 | 42 | | | 34 | 26 | | |
| *M classification* | | | | | | | | | |
| M0 | 114 | 51 | 63 | 0.546 | 0.693 | 64 | 50 | 1.725 | 0.239 |
| M1 | 6 | 2 | 4 | | | 5 | 1 | | |

isolated by incubating with 15 mM EDTA at 37 °C for 20 min, as we described previously.[17] The cell pellets were lysed with RIPA lysis buffer immediately or stored at −80 °C for immunoblotting analysis. The cell pellet was washed twice with ice-cold PBS and lysed with lysis buffer. 30 μg of protein was loaded and separated in 12% SDS–PAGE gel and transferred to polyvinylidine difluoride membranes (Millipore, Bedford, MA). The following antibodies were used to probe the alterations of protein: anti- RXRα, β-catenin (Santa Cruz Biotechnology, Santa Cruz, CA), Signal was detected by enhanced chemoluminescence techniques (Amersham Life Science, Piscataway, NJ), as described previously.[16] β-actin or α-tubulin (Sigma, St. Louis, MO) was used as loading control.

### 2.4. Immunohistochemistry staining

The sections were deparaffinised, rehydrated in serially graded ethanol and heated in citric buffer (pH 6.0) once for 5 min in a microwave oven for antigen retrieval. They were then washed with distilled water, blocked with 3% hydrogen peroxide and incubated with the primary antibody at 4 °C for 12 h. After washing with a 0.01 mol/L concentration of phosphate buffered saline (PBS), the sections were incubated with EnVision-HRP secondary antibody(Dako) for 30 min 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. The working concentration of primary antibody for the detection of RXRα and β-catenin (Santa Cruz Biotechnology, Santa Cruz, CA) was 1:200 and 1:100, respectively.

### 2.5. Evaluation of immunohistochemistry staining

Immunohistochemical staining was independently assessed by two observers who had no knowledge of the clinicopathologic data. The staining of β-catenin was scored according to Maruyama's method.[18] 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 ectopic expression. The degree of RXRα immunostaining was based on both the proportion of positively stained tumour cells and intensity of staining. The proportion of positively stained tumour cells was scored as follows: 0 (no positive tumour cells), 1(<10% positive tumour cells), 2 (10–50% positive tumour cells), and 3 (>50% positive tumour 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 × the proportion score. We evaluated the expression of RXRα in colorectal specimen by determining the staining index, with scores 0, 1, 2, 3, 4, 6, or 9. The staining index score of 4 (the cutoff point) was used to distinguish between low and high expression of RXRα.

### 2.6. Methylation analysis

Genomic DNA was extracted from four pairs of fresh tissues with CRC and ANT using the DNA Extraction Kit (Qiagen, Hilden, Germany). To investigate the methylation status of RXRα, methylation-specific polymerase chain reaction (MSP) with primers specific for the methylated and unmethylated alleles of each gene after treatment of the genomic DNA with sodium bisulfite using EpiTect® Bisulfite Kit (Qiagen, Hilden, Germany) was performed. The unmethylation and methylation-specific primer sequences for RXRα were same as Lee et al. reported.[19] The amplification condition included the initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30s, primer annealing at 53 and 57 °C for 30s for unmethylation primers and methylation primers, respectively, and extension step at 72 °C for 45s, followed by the final extension step at 72 °C for 10 min. The expected amplification product by PCR in 2% agarose gel was 151 bp. Each MSP was repeated once to confirm the results.

### 2.7. Statistical analyses

All statistical analyses were performed using SPSS 16.0 statistics software. Chi-square test and Fisher exact test were used to compare the levels of RXRα and β-catenin expression with different groups and various clinicopathologic parameters. The correlation of β-catenin and RXRα expression was analysed by Spearman's correlation coefficients. $P < 0.05$ was set to be statistically significant.

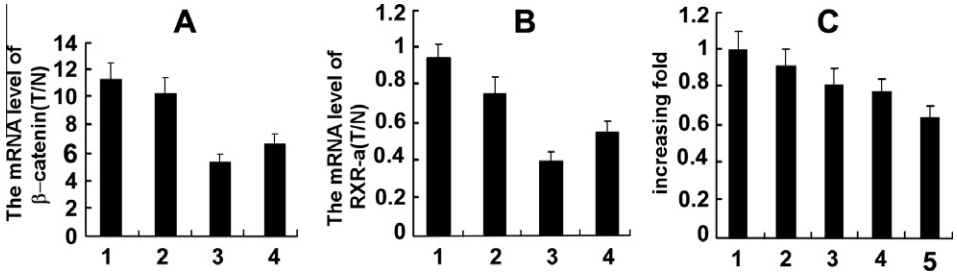


**Fig. 1 – β-Catenin and RXRα mRNA expression in CRC by real-time PCR. (A) β-Catenin expression was elevated in 4 cases of CRC compared with that in adjacent non-tumour tissues (ANT); (B) RXRα expression was down-regulated in CRC compared with ANT; (C) RXRα expression was decreased in well (column 2), moderately (column 3), poorly CRC(column 4) and CRC with lymph node metastasis (column 5) in comparison to ANT (column 1).**

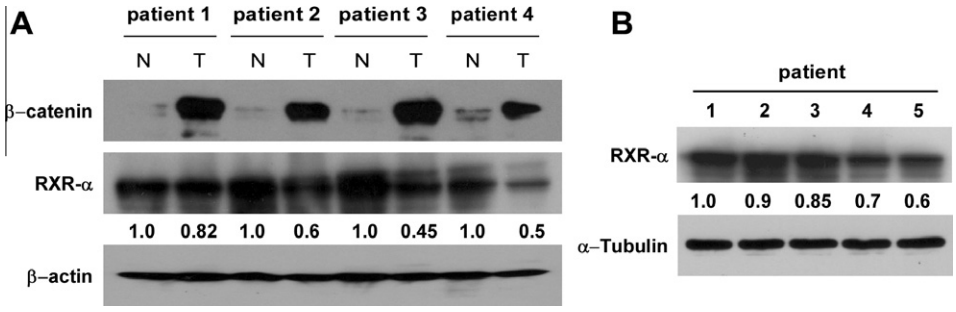


**Fig. 2 – β-Catenin and RXRα expression in CRC by Western blot. (A) β-Catenin expression was elevated in 4 cases of CRC compared with that in ANT, however, RXRα expression was down-regulated in CRC compared with ANT; The relative quantification of bands in Western blot was a ratio neutralised to β-actin. (B) RXRα expression was downregulated in well (column 2), moderately (column 3), poorly CRC(column 4) and CRC with lymph node metastasis (column 5) in comparison to ANT (column 1). The relative quantification of bands in Western blot was a ratio neutralised to α-tubulin.**

## 3. Results

### 3.1. *β-Catenin and RXRα mRNA expression in fresh CRC and ANT*

Real-time PCR was performed in 4 pairs of fresh CRC and ANT. Our results showed that β-catenin mRNA expression was higher in all CRC compared with that in the respective ANT (Fig. 1A). However, RXRα mRNA expression was reduced in all CRC compared with ANT (Fig. 1B). To further determine whether RXRα expression was related to histological differentiation and metastasis of CRC, real-time PCR was performed in 1 ANT and 4 CRC tissues, which included well, moderately, poorly differentiated and CRC with lymph node metastasis each. The results showed that decreased RXRα mRNA expression was related to histopathological differentiation degree and metastasis status of CRC (Fig. 1C).

### 3.2. *β-Catenin and RXRα protein expression in fresh CRC and ANT*

To determine β-catenin and RXRα protein expression in 4 such pairs of fresh CRC and ANT, the results consisted with mRNA expression of β-catenin and RXRα using western blot analysis. β-catenin and RXRα expression was increased and decreased in all CRC compared with ANT, respectively (Fig. 2A). Moreover, suppression of RXRα expression was related to histopathological differentiation degree and metastasis status of CRC (Fig. 2B).

### 3.3. *β-Catenin and RXRα expression in paraffin-embedded CRC, adjacent non-tumour mucosa and lymph node metastatic CRC*

β-Catenin protein can be detected in cell membrane, cytoplasm, and nuclei. The expression of β-catenin in cell membrane was regarded as normal, and its expression in cytoplasm and nuclei was defined as ectopic expression. Representative immunohistochemistry staining of β-catenin in adjacent non-tumour mucosa (ANM) and CRC was shown in Fig. 3A. Positive β-catenin staining in cell membrane, cytoplasm and nuclei was 53(44%), 117(97%), and 75(62%) cases of CRC, but 120(100%), 79(65%), and 0 case of ANM, respectively. The difference was statistically significant ($P < 0.001$, Fig. 4A). The results demonstrated that β-catenin expression was aberrant in CRC compared with ANM. In addition, ectopic β-catenin expression was significantly higher in CRC with lymph node metastasis than that in CRC without lymph node metastasis ($P = 0.002$, Fig. 4B). However, there was no significant difference of ectopic β-catenin expression between primary CRC and lymph node metastatic CRC ($P = 0.685$, Fig. 4C).

RXRα positive signal was located in the nuclei. Low and high RXRα expression was 69(57%) and 51(43%) in CRC, but 42(35%) and 78(65%) in ANM, respectively. RXRα expression was significantly lower in CRC than ANM ($P < 0.001$, Fig. 4D). In addition, RXRα expression was significantly lower in CRC with lymph node metastasis than that in CRC without lymph node metastasis ($P = 0.002$, Fig. 4E). However, there was no significant difference of RXRα expression between primary CRC and lymph node metastatic CRC ($P = 0.35$, Fig. 4F). Representative immunohistochemistry staining of RXRα in NTM and CRC were shown in Fig. 3B.

### 3.4. *The relationship between β-catenin and RXRα expression and the clinicopathologic features of CRC*

Table 1 revealed that β-catenin ectopic expression was closely associated with histological differentiation ($P < 0.001$), N classification ($P = 0.002$), whereas β-catenin ectopic expression was not related to gender, age, UICC stage, T classification, and M classification. Meanwhile, RXRα expression was significantly related to histological differentiation ($P < 0.001$), UICC stage ($P = 0.013$), and N classification ($P = 0.001$). However, no significant association of RXRα expression with gender, T classification and M classification ($P > 0.05$) was found. The results suggest that β-catenin ectopic expression and RXRα expression were closely related to histological differentiation and progression of CRC.

### 3.5. *The correlation between β-catenin ectopic expression and RXRα expression*

To determine the correlation between β-catenin ectopic expression and RXRα expression level, Spearman correlation

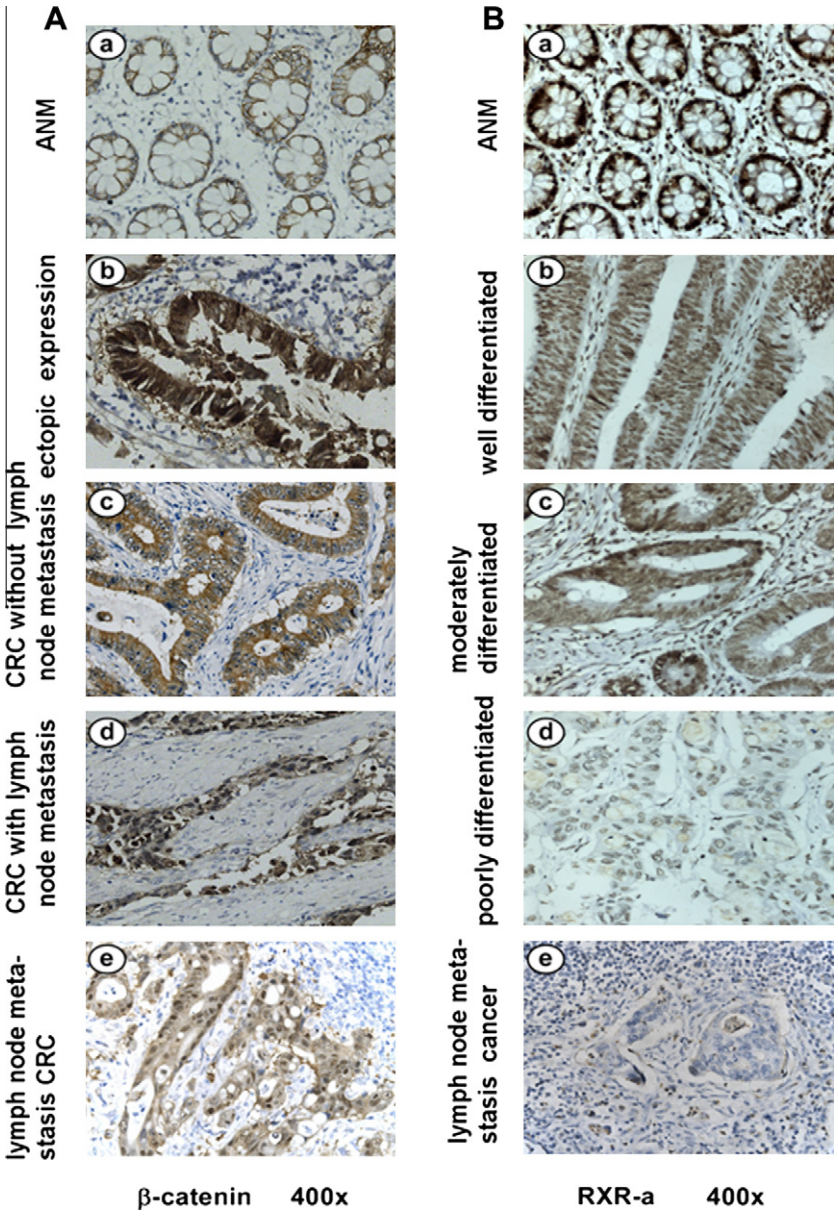


**Fig. 3 – β-Catenin and RXRα expression in CRC by immunohistochemical staining. (A) β-Catenin ectopic expression was shown in CRC(b), CRC without lymph node metastasis(c), CRC with lymph node metastasis(d), and lymph node metastatic carcinoma of CRC (e) compared with that in ANM(a). (B) Reduced RXRα expression in well (b), moderately (c), poorly CRC (d) and lymph node metastatic carcinoma of CRC (e) in comparison to ANT (a). Immunohistochemical staining × 400.**

analysis was performed. There was significantly correlation between β-catenin ectopic expression and low RXRα expression (pearson's $R = -0.155$, $P = 0.032$, Table 2).

### 3.6. *Methylation analysis of RXRα gene in fresh CRC and ANT tissues*

The methylation status of the RXRα genes was determined in 4 pairs fresh CRC and ANT tissues using MSP. As shown in Fig. 5, unmethylated bands were detected in both non-malignant and malignant tissues, thus confirming the integrity of the DNA in these samples. Methylation of the RXRα gene was detected in all 4 pairs of CRC and ANT tissues.

## 4. Discussion

The Wnt/β-catenin/TCF signalling pathway plays an important role in gene expression, cell adhesion, tissue development and initiation of carcinogenesis.[20,21] It is well known that β-catenin is regulated by two APC-dependent proteasomal degradation pathways—GSK3β-regulated pathway involving the Apc/Axin complex and a p53-inducible pathway involving Siah-1.[20–23] Recently, several lines of evidence

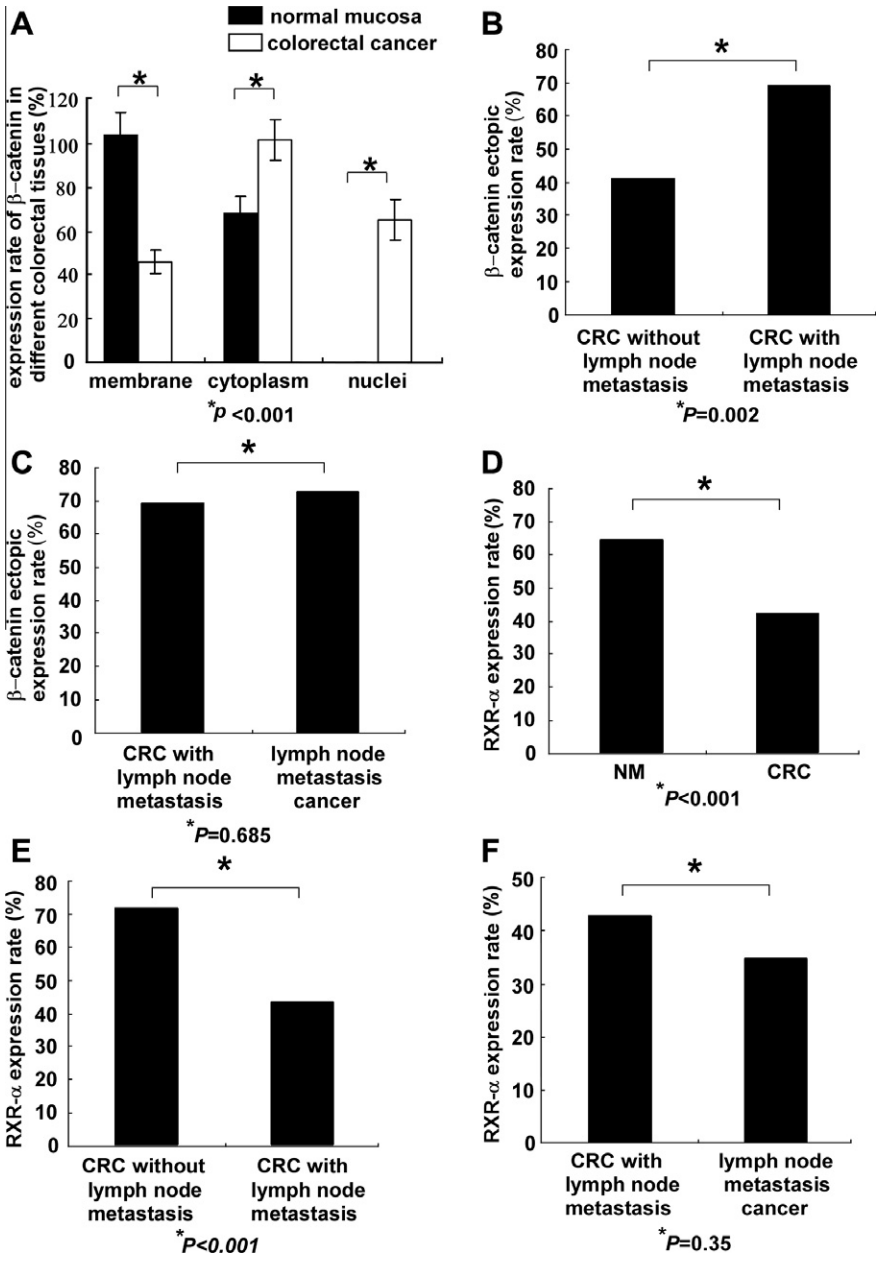


**Fig. 4 – β-Catenin and RXRα expression in CRC by immunohistochemical staining. (A) β-Catenin expression in cell distribution of CRC compared with that in ANM(*P < 0.001). (B) β-Catenin ectopic expression was elevated in CRC with lymph node metastasis compared with CRC without lymph node metastasis (*P = 0.002); (C)There was no significant difference between β-catenin ectopic expression in primary CRC and respective lymph node metastasis (*P = 0.685). (D) RXRα expression was downregulated in CRC compared with ANM (*P < 0.001). (E) RXR-α expression was downregulated in CRC with lymph node metastasis compared with CRC without lymph node metastasis (*P < 0.001). (F) There was no significant difference between RXRα expression in primary CRC and respective lymph node metastasis (*P = 0.35).**

showed that nuclear receptors affected β-catenin/TCF/LEF-mediated gene transcription and β-catenin protein. For example, retinoid-activated RAR acts as a potent repressor of β-catenin/TCF signalling in retinoid-sensitive colorectal cancer cells[24–26], and activation of the vitamin D receptor with its metabolite ligand, 1α,25(OH)2 vitamin D3, could repress Wnt/β-catenin/TCF signalling.[27–29] Retinol increases β-catenin-RXRα binding leading to the increased proteasomal degradation of β-catenin and RXRα.[30] Our previous work in the first time demonstrated that RXRα overexpression directly inhibited endogenous and exogenous β-catenin transcriptional activity and expression in the absence of RXR agonist. However, the inhibition was abrogated by targeted RXRα small RNA interfering.[16] Further study showed that RXRα directly interacted with β-catenin using both mammalian two-hybrid assay *in vivo* and immunoprecipitation.[16] Because nuclear receptors can interact with β-catenin under certain circumstances we initially hypothesised that retinol-mediated

**Table 2 – The correlation between RXRα expression level and β-catenin ectopic expression in CRC.**

| | RXR-α expression level | |
|---|---|---|
| | Low expression | High expression |
| *β-catenin ectopic expression* | | |
| Cytoplasm | 68 | 49 |
| Nuclei | 55 | 20 |
| *P* value | 0.032 | |

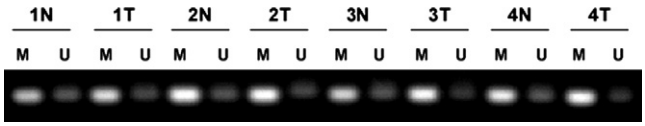


**Fig. 5 – The methylation status of RXRα gene was analysed in 4 pairs of fresh CRC and ANT tissues using MSP method. Lane U, amplified product obtained using unmethylation primers; Lane M, amplified product obtained using methylation primers.**

trans-repression of β-catenin signalling involved a direct RXR/β-catenin interaction and interference with TCF mediated transcription. In order to investigate β-catenin and RXRα expression in human ANT, CRC and lymph node metastasis of CRC, we detected β-catenin and RXRα expression at both mRNA and protein levels using Real-time PCR and western blot analyses. The results showed that β-catenin and RXRα expression at both mRNA and protein levels increased and decreased in CRC compared with ANT, respectively. Furthermore, decreased RXRα expression was related to histological differentiation degree and lymph node metastasis status of CRC. To further confirm such results, 120 cases of primary CRC, 60 cases of lymph node metastatic carcinoma of CRC were used to detect β-catenin and RXRα expression using immunohistochemistry. The results showed that β-catenin expression was aberrant in CRC compared with ANM. In addition, ectopic β-catenin expression was significantly higher in CRC with lymph node metastasis than that in CRC without lymph node metastasis. however, RXRα expression was significantly lower in CRC than ANM. Moreover, RXRα expression was significantly lower in CRC with lymph node metastasis than that in CRC without lymph node metastasis. The results of the current work have confirmed our previous work in human CRC tissue.

To determine the relationship between β-catenin and RXRα expression and the clinicopathological features of CRC, our data showed that low RXRα or β-catenin ectopic expression was significantly associated with histological differentiation and lymph node metastasis. In addition, low RXRα expression was closely related to UICC stage of CRC. The results suggest that low RXRα expression or β-catenin ectopic expression is closely associated with pathogenesis and progression of CRC. Moreover, there was significant correlation between low RXRα expression and β-catenin ectopic expression in CRC. The data was identical to our previous work which was performed in CRC cell lines.[16] Recently, methylation-associated downregulation of the RXRG gene underlying the impaired expression of RXRs in lung cancer may play a role in the carcinogenesis of non-small cell lung cancer.[31] Methylation of RARβ isoforms has been reported in human colon cancer.[32] Volate et al. reported a low concentration of green tea was sufficient to desilence RXRα and inhibit intestinal tumourigenesis in the Apc(Min/+) mouse.[33] So epigenetic modulation of RXRα may be a novel strategy for prevention and treatment of CRC. We analysed the methylation status of RXRα gene in 4 pairs of fresh CRC and ANT tissues using MSP method and found that methylation of the RXRα gene was detected in all 4 pairs of CRC and ANT tissues. As for this issue, it needs further study in a large series of CRC samples.

In summary, our data firstly showed that β-catenin and RXRα expression increased and decreased in CRC compared with ANT, respectively. RXRα expression was downregulated in higher histological grade CRC. There was significant correlation between low RXRα expression and β-catenin ectopic expression in CRC. Low RXRα expression or β-catenin ectopic expression was closely associated with pathogenesis and progression of CRC.

## Conflict of interest statement

None declared.

## Acknowledgments

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