

REPORT

Oncogenic retinoic acid receptor γ knockdown reverses multi-drug resistance of human colorectal cancer via Wnt/ β -catenin pathway

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ABSTRACT

Retinoic acid receptor γ (RAR γ), a unique member of the nuclear receptor superfamily, plays an important role in the progression of several cancers such as hepatocellular carcinoma, esophageal cancer, and cholangiocarcinoma. However, little is known about the regulatory mechanism of the RAR γ expression in colorectal cancer (CRC) progression. In the present study, we found that RAR γ was frequently overexpressed in human CRC specimens and CRC cell lines, and it mainly resided in the cytoplasm in CRC specimens. Tissue microarrays showed that RAR γ indicated vital clinical significance in CRC. RAR γ knockdown neither affected CRC cell proliferation nor blocked the cell cycle of CRC cells. However, RAR γ knockdown increased the sensitivity of CRC cells to chemotherapeutics through downregulation of multi-drug resistance 1 (MDR1). Further studies suggested that RAR γ knockdown resulted in downregulation of MDR1, in parallel with suppression of the Wnt/ β -catenin pathway. Moreover, a significantly positive association between RAR γ and MDR1 was demonstrated in CRC tissue microarrays. Collectively, these results suggested that overexpression of RAR γ contributed to the multidrug chemoresistance of CRC cells, at least in part due to upregulation of MDR1 via activation of the Wnt/ β -catenin pathway, indicating that RAR γ might serve as a potential therapeutic target for chemoresistant CRC patients.

ARTICLE HISTORY

Received 14 November 2016
Revised 30 December 2016
Accepted 10 February 2017

KEYWORDS

Retinoic acid receptor γ ;
multi-drug resistance 1;
 β -catenin; chemoresistance;
colorectal cancer

Introduction





Colorectal cancer (CRC) is the third malignant tumors with the third most common cause of cancer-related mortality around the world.¹ CRC is characterized by poor prognosis of early stage, early metastasis, remarkably high malignancy, easy to relapse of surgical removal and multidrug resistance (MDR).² Therefore, chemoradiotherapy offers the unique possibility of cure for the majority of CRC patients. However, radiation therapy and currently available chemotherapeutics have limited efficacy and can barely improve patient survival because of MDR.³ Hence, there is imperative to have a better understanding of the molecular mechanism involved in CRC progression, which is important for development of novel therapeutic strategies for the treatment of CRC.

Retinoic acid receptor γ (RAR γ) is a member of the nuclear receptor superfamily that is essential in embryonic development, maintenance of differentiated cellular phenotypes, metabolism, and cell death.⁴ There are 3 RAR subtypes, α , β , and γ . Among them, RAR γ plays unique and uncharacterized roles in many physiologic processes including carcinogenesis. RAR γ is a critical physiologic and pharmacological regulator of the balance between haematopoietic stem cell self-renewal and differentiation.⁵ It interacts with Wnt/ β -catenin to regulate chondrocyte function and matrix turnover.⁶ Retinoic acid

(RA), a RAR γ ligand, increases death of SH-SY5Y neuroblastoma cells and induces apoptosis of pancreatic cancer cells depending on RAR γ ,^{7,8} indicating the suitability of targeting RAR γ for cancer therapy. Consistently, the oncogenic potential of RAR γ has been demonstrated. RAR γ is overexpressed in multiple human cancers such as hepatocellular carcinoma, esophageal cancer, and cholangiocarcinoma and plays vital roles in promoting the progression of tumors through multiple signaling pathways including PI3K/Akt, NF- κ B, and Wnt/ β -catenin.^{9–11} However, the expression profile of RAR γ in CRC and its function remained unknown.


Multidrug resistance (MDR) is a major obstacle to develop effective therapeutic drugs for the treatment of cancers.¹² The typical mechanism contributing to MDR is mainly associated with reduced intracellular drug concentration and an increased drug efflux.¹³ Upregulation of drug transporters results in an active efflux, thus inducing cancer cells to resist chemotherapeutic drugs.¹⁴ ATP-binding cassette transporters such as Multidrug resistance 1 (MDR1/P-gp/ ABCB1),¹⁵ have been implicated in the multidrug resistance of most human tumors including CRC and constitute an important cause of therapeutic failure.^{12,16–18}

In the present study we demonstrated that RAR γ is frequently overexpressed in human CRC tissues and CRC cell lines when compared with their respective controls. RAR γ

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knockdown increased the sensitivity of CRC cells to chemotherapeutic drugs. Moreover, RAR γ knockdown suppressed MDR1 expression in CRC cells via the Wnt/ β -catenin pathway. In addition, there is a significant correlation between RAR γ level and the MDR1 expression in CRC tissues. Our findings suggested that RAR γ might serve as a new molecular target for chemoresistant CRC patients.

Materials and methods

Reagents

Methyl thiazolyl tetrazolium (MTT) and propidium iodide (PI) were purchased from Sigma (St. Louis, MO, USA). Vincristine sulfate (VCR) and 5-fluorouracil (5-FU) were purchased from Sigma-Aldrich (Indianapolis, IN, United States). Polyclonal antibodies against RAR γ and MDR1 were from Abcam Ltd. (Cambridge, United Kingdom). Goat anti-rabbit and anti-mouse secondary antibodies conjugated to horseradish peroxidase and Lipofectamine 2000 from Invitrogen (Carlsbad, CA, USA). PVDF membrane was from Millipore (Billerica, MA, USA). The EliVision Plus kit was obtained from Maixin Bio (Fuzhou, China).

Patients and tumor specimens

Tumorous and their adjacent noncancerous colorectal cancer tissues were collected from patients who underwent surgery at the First Affiliated Hospital of Xiamen University. Written informed consent was obtained from each patient and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Institute Research Ethics Committee of the First Affiliated Hospital of Xiamen University. Fresh surgical samples from colorectal cancer tissues were collected between 2011 and 2014.

Construction of lentivirus-based shRNA expression vector

PLL3.7 vector mediated shRNA was performed by constructing RAR γ targeting sequence (5'-GCTACCAAGTGCATCATCA-3') into pLL3.7-neo vector to generate pLL3.7-shRAR γ . The sequence (5'-TTCTCCGAACGTGTCACGT-3') was used for control shRNA. Lentivirus packaging and infection were performed as described previously.¹⁹

Cell culture and stable cell lines

HT29 and HCoEpiC were cultured in RPMI1640. HCT116, RKO and SW480 were grown in Dulbecco's modified Eagle's medium (DMEM). Both medium was supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 U/mL streptomycin at 37°C in a 5% CO₂ incubator. After lentivirus infection with pLL3.7-shRAR γ or with pLL3.7-shCtrl, stably transfected HT29, HCT116 and RKO cells were established by treatment with geneticin (250 μ g/mL) for 3 weeks.

Cell proliferation assay

Cell proliferation was analyzed by MTT assay, as described previously.²⁰ A total of 2×10^3 cells were seeded in each

well of 96-well plates, and MTT was added to each well every 24 h. The plates were incubated for 4 h before addition of 100 μ L DMSO. The absorbance was measured at 490 nm with a microplate reader (Model 680; Bio-Rad, Hercules, CA, USA)

Cell cycle analysis

ShRAR γ -RKO and shCtrl-RKO cells were synchronized by serum starvation 24 h and then cultured by an exchange of 10% fetal bovine serum for 24 h. And then the cells were harvested, and fixed in 75% ethanol at 4°C for 30 min. Cells were incubated with RNase A at 37°C for 30 min, and then stained with propidium iodide for 30 min. Cell cycle was measured by flow cytometry. The data were analyzed with the ModFit 3.3 (Verity Software House, Topsham, ME, USA) software.

Quantitative PCR

Quantitative real-time PCR (qPCR) was performed using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control. The primers for PCR reactions are as follows: GAPDH, forward: GAACTTCTG GATGCTGGTG, reverse: TACGTGAATGTGGCCTGT; RAR γ , forward: AAAACTGTATCATCAACAAGG, reverse: CTTACCTCTTCTCTTCTTG. MRP primers are as described previously.²¹

Western blotting

Equal amounts of protein lysates were electrophoresed on 10% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membrane. The membrane was then incubated with primary and secondary antibodies, and the signal was finally detected using an enhanced chemiluminescence (ECL) system.

IHC (Immunohistochemistry)

Paraffine-embedded human CRC tissue sections were immunostained with antibody against RAR γ (1:200) or MDR1 (1:200) and detected with corresponding secondary antibody. The slides were stained with DAB for 5 min and then costained with hematoxylin to visualize nuclei.

Dual-luciferase reporter assays

The cells (1.0×10^4 cells / well) were seeded in 96-well plates for 24 h before transfection. Then cells in each well were cotransfected with 100 ng pTOPFlash or pFOPFlash reporter plasmid and 20 ng Renilla luciferase expression vector using Lipofectamine 2000 for another 24 h and subsequently with Wnt3a (50 ng/mL) for 6 h. The activities of both firefly luciferase and renilla luciferase were measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). The firefly luciferase activity was normalized to renilla luciferase activity.

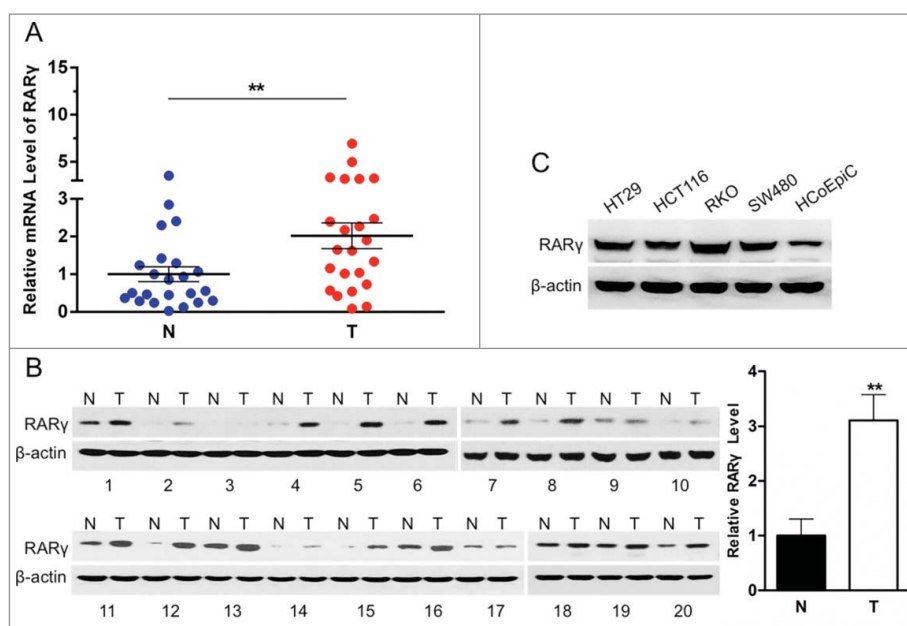


Figure 1. RAR γ is overexpressed in human CRC tissues and cell lines. (A) Expression of RAR γ mRNA in 23 human CRC samples and their surrounding non-tumorous tissue samples. (B) Expression of RAR γ protein in 20 human CRC specimens and their adjacent specimens (Left). Quantitative analysis of RAR γ protein in CRC specimens (Right). (C) Western blot analysis of expression of RAR γ protein in normal colon epithelium cells and 4 CRC cell lines. ** $P < 0.01$.

Statistical analysis

Statistical analysis was performed by using GraphPad Prism 6 (San Diego, CA, USA). Data are represented as means \pm SD from at least 3 independent experiments. One-way analysis of variance (ANOVA) or *t*-test was used for comparison of 2 and more than 2 data sets. P value ≤ 0.05 was considered statistically significant.

Results

RAR γ expression is frequently upregulated in human CRC tissues and cell lines

To evaluate the expression of RAR γ in CRC, we performed qPCR and western blotting to assess the mRNA and protein level of RAR γ in a set of 23 tumor and adjacent non-tumorous colorectal tissues and protein in paired 20. The mRNA and protein levels of RAR γ were significantly upregulated in tumor specimens versus the surrounding non-tumorous colorectal tissues (Fig. 1A, B). In addition, RAR γ expression was also significantly increased in CRC cell lines such as HT29, HCT116, RKO, and SW480 compared with normal colonic epithelium HCoEpiC (Fig. 1C). Therefore, overexpression of RAR γ in CRC specimens as well as in CRC cell lines suggested that RAR γ might play a role in CRC progression.

Clinical significance of RAR γ in CRC

To further assess expression of RAR γ , immunohistochemistry (IHC) was used to analyze RAR γ expression on 90 paraffin-embedded CRC tissues and paired paracarcinoma tissues on tissue microarrays (TMAs). The IHC results showed that RAR γ , predominantly present in the cell cytoplasm, was strongly stained in CRC tissues but weakly or not stained in paired adjacent colorectal tissues (Fig. 2A). The staining

intensity of RAR γ protein was categorized as high (2+, 3+) and low (\pm , +) (Fig. 2B), and the high-expression rate of RAR γ protein in CRC tissues (73.3%) was much higher than that in adjacent non-tumorous colorectal tissues (20%) ($P < 0.001$) (Table 1).

The clinical data in Table 2 show that RAR γ overexpression was significantly associated with pathological differentiation, T classification and clinical stages ($P < 0.05$), whereas no association was found with age, sex, tumor size, lymph node metastasis and distant metastasis ($P > 0.05$). Furthermore, the postoperative overall survival rate in patients with high RAR γ expression was lower than that in patients with low RAR γ expression ($P < 0.05$) (Fig. 2C). These results indicated the clinical significance of RAR γ in the diagnosis and prognosis of CRC patients.

RAR γ knockdown enhances the sensitivity of CRC cells to chemotherapeutics

To investigate the role of RAR α in the growth of CRC cells, MTT assay was performed and showed that stable knockdown of RAR γ in these cells resulted in no significant changes in cell proliferation (supplementary Fig. 1A), while cell cycle analysis was obtained consistent results. The cell cycle of RAR γ knockdown cells did not arrest in any phase of the cell cycle (supplementary Fig. 1B). These data demonstrated that RAR γ knockdown did not affect CRC cell growth.

To establish stable RAR γ knockdown cells for further study, RAR γ -specific shRNA, but not control shRNA, efficiently reduced the levels of the endogenous RAR γ protein in these cells (Fig. 3A). We further explored whether RAR γ modulates the resistance of CRC to chemotherapeutic drugs. RAR γ knockdown enhanced the sensitivity of all 3 CRC cell lines to L-OHP, 5-FU and VCR (Fig. 3B). These data indicated that RAR γ plays an important role in cancer drug resistance.

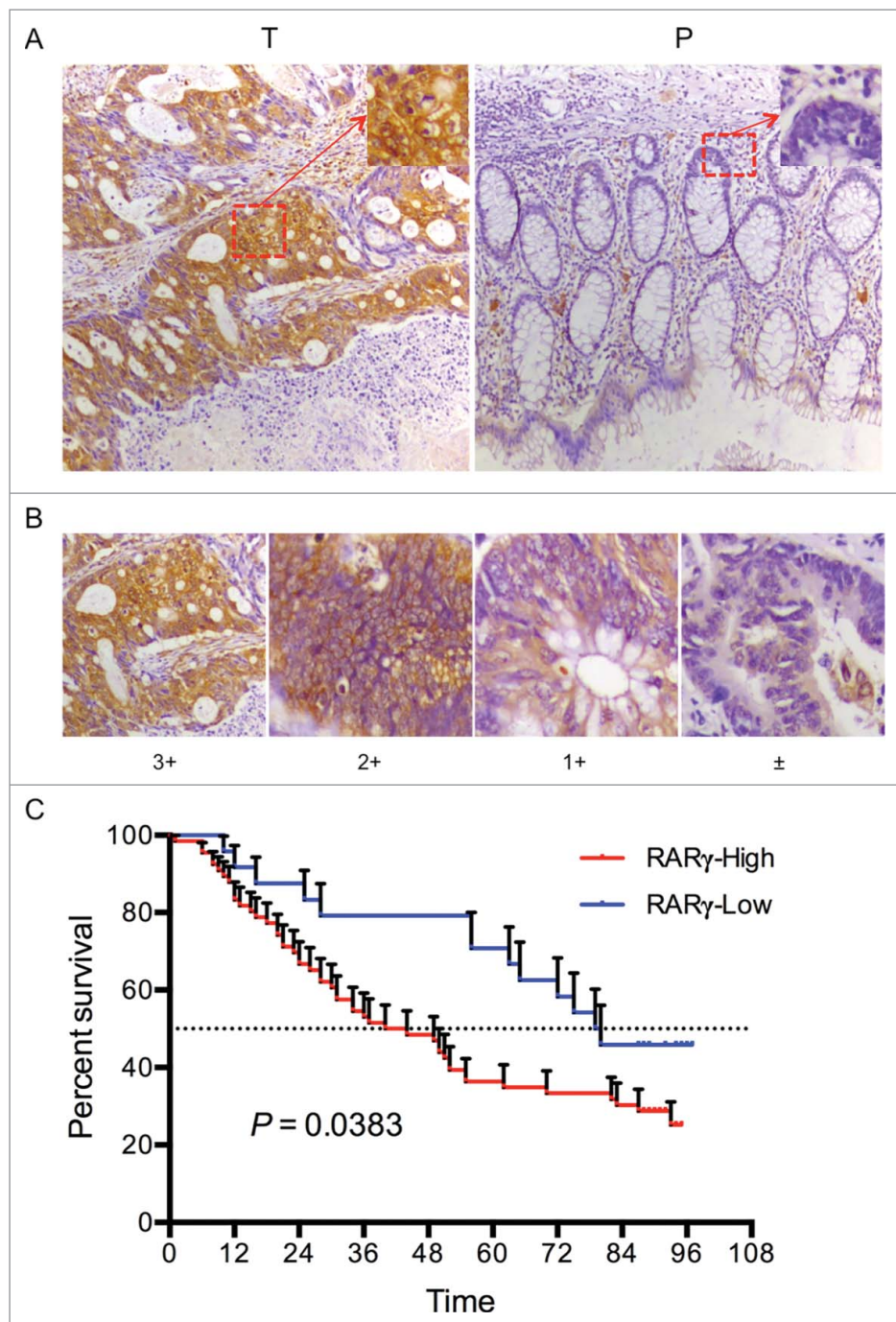


Figure 2. Clinical significance of RAR γ in CRC. (A) Immunohistochemical analysis of RAR γ expression on tissue microarrays. T: tumor tissue, P: paracarcinoma tissue. (B) Representative staining intensity of tumors classified as 4 different grades. (C) Correlations of the RAR γ protein expression with overall survival rate.

RAR γ upregulates MDR1 via Wnt/ β -catenin pathway

To determine the mechanism by which RAR γ knockdown sensitizes CRC cells to chemotherapeutics, the mRNA levels of the MDR associated proteins were assessed. RAR γ knockdown

Table 1. Distribution of RAR γ classifications on CRC and paracarcinoma tissues.

Tissue type	N	RAR γ stain grades				χ^2	P
		–	+	++	+++		
Tumor	90	3	21	31	35	57.43	< 0.0001
Paracarcinoma	90	26	46	13	5		

resulted in the repression of MDR1, but not MRP1, MRP2 or MRP3 mRNA (supplementary Fig. 1C). Indeed, the protein expression of MDR1 decreased in RAR γ knockdown CRC cells compared with the control cells (Fig. 4A). In addition, western blotting analysis showed that the expression of β -catenin was decreased in RAR γ knockdown CRC cells, along with phosphorylation of β -catenin at ser33/37 was increased. Moreover, Wnt3a-induced accumulation of β -catenin markedly decreased in RAR γ knockdown CRC cells (Fig. 4B). Wnt3a-induced activity of the TCF/LEF reporter gene was attenuated by RAR γ knockdown (Fig. 4C). Furthermore, RAR γ -induced increase of MDR1 protein was abolished by siRNA against β -catenin

Table 2. Relationships between RAR γ and clinical features in CRC.

Features	n	RAR γ		χ^2	P
		Low	High		
Age				0.051	0.8216
< 60	21	6	15		
≥ 60	69	18	51		
Sex				0.050	0.8238
female	43	11	32		
male	47	13	34		
Pathological differentiation				27.04	0.0001*
I, I-II	17	13	4		
II	38	7	31		
II-III, III	35	4	31		
Tumor diameter				0.6851	0.7100
d \leq 4	28	9	19		
4 < d < 6	30	8	22		
d \geq 6	31	7	24		
T classification				8.925	0.0115*
T2	11	7	4		
T3	62	14	48		
T4	17	3	14		
N classification				0.5830	0.4451
N0	58	17	41		
N+	32	7	25		
M classification					
M0	88	23	65		
M1	2	1	1		
Clinical stages				12.81	0.0017*
1	12	7	5		
2	48	15	33		
3	30	2	28		

(Fig. 4D). Collectively, these data demonstrated that RAR γ might serve as an essential coactivator for Wnt/ β -catenin pathway activation and thus enhances its transcriptional activity for MDR1.

RAR γ associates with chemoresistance in CRC specimens

To determine whether RAR γ -regulated MDR1 expression in CRC cell lines has clinical implications, tissue microarrays from 90 patients with CRC who had undergone resection were examined via immunostaining with RAR γ and MDR1 antibodies. Tissue microarray analysis revealed a strong correlation between RAR γ and MDR1 levels (Fig. 5A). Statistical *t*-test indicated a very significant ($P < 0.0001$) positive correlation ($r = 0.6148$) (Fig. 5B). These data suggested that RAR γ might contribute to chemoresistance of CRC patients.

Discussion

Like other nuclear receptors, RAR γ is known to play vital genomic roles through regulating the transcription of target genes by binding to DNA response elements.⁴ Accumulating evidence, however, indicated that RAR γ may also have extranuclear actions, which shows non-genomic regulatory effects. Aberrant RAR γ expression was been considered as a common

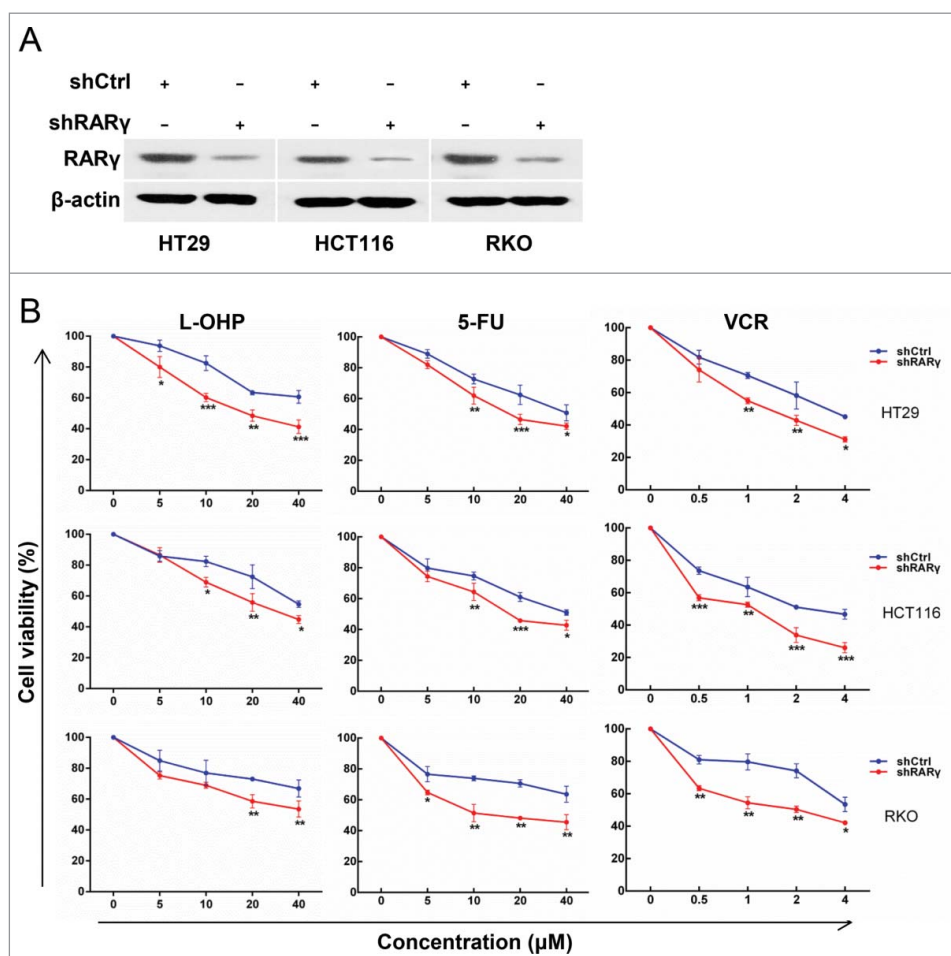


Figure 3. Effect of RAR γ knockdown on the sensitivity of CRC cells to chemotherapeutic drugs. (A) RAR γ knockdown confirmed by western blot analysis. (B) CRC cells treated with L-OHP, 5-FU or VCR for 24 hours, and then cell viability measured by MTT assay.

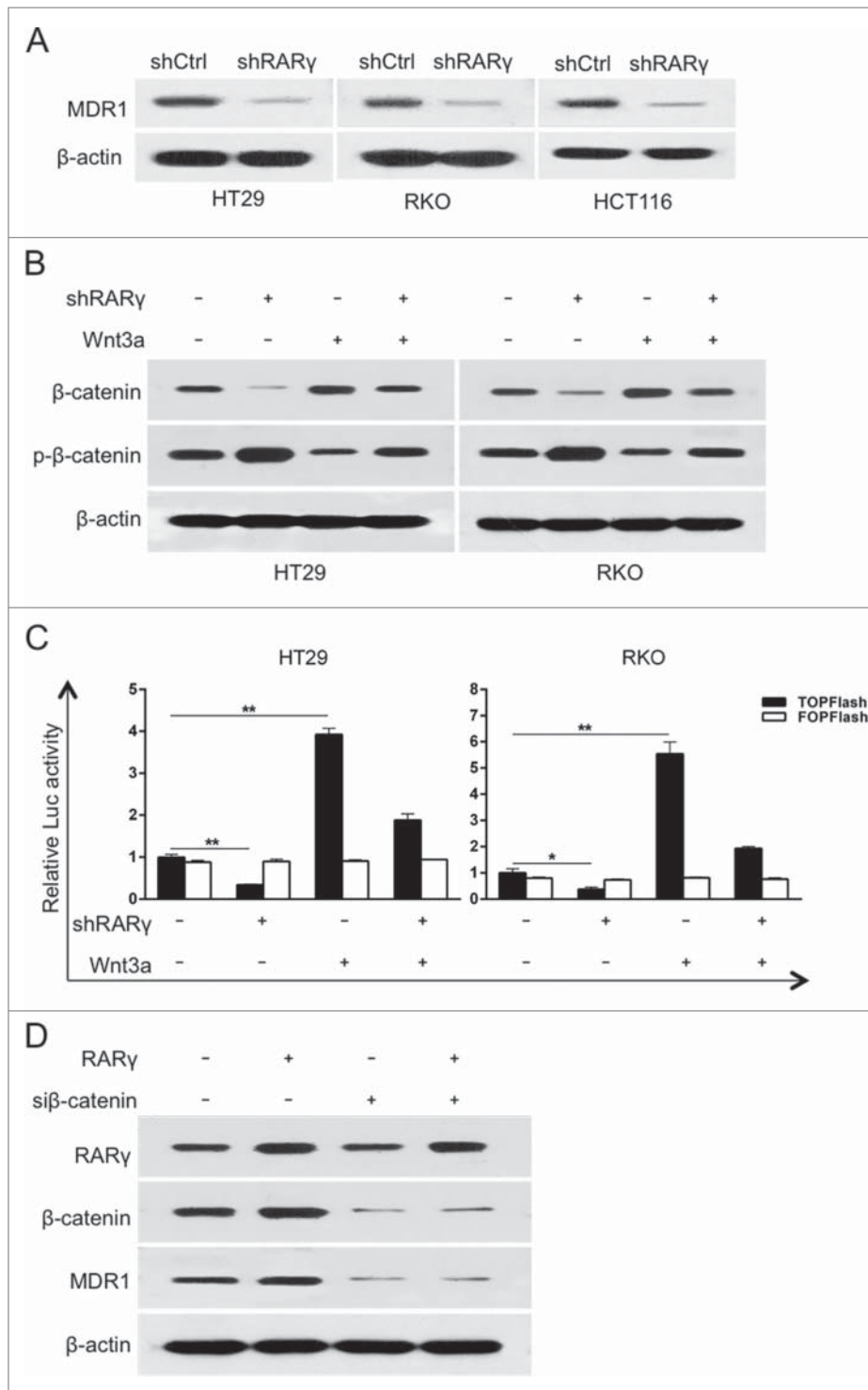


Figure 4. RAR γ knockdown inhibited the Wnt/ β -catenin pathway. (A) The protein levels of MDR1 in RAR γ -knockdown CRC cells. (B) Protein levels of phosphorylated β -catenin (p- β -catenin) and β -catenin in CRC cells assessed by western blot. (C) T-cell factor / lymphoid enhancing factor (TCF/LEF)-responsive luciferase activity assessed by dual-luciferase reporter assay. A representative experiment of 3 independent experiments is shown. (D) Western blot analysis of the indicated proteins after transfection for 24 h.

phenomenon in multiple human cancers. But, the expression profile and the role of RAR γ in CRC remained elusive. In the present study, we found that the overexpression of RAR γ was detected in most of the CRC tissues and all CRC cell lines examined, suggesting that RAR γ may play a role in CRC progression. Our results are consistent with previous studies regarding the expression profile of RAR γ in hepatocellular carcinoma, esophageal cancer, and cholangiocarcinoma.⁹⁻¹¹

Additionally, we found that RAR γ was abnormally expressed in the cytoplasm of human CRC specimens, which is in concert with previous studies that RAR γ often resided in the cytoplasm of cancer cells.^{9,22} Arguably the most fundamental trait of cancer cells involves their capability to sustain proliferation signaling.²³ Our data presented here showed that RAR γ knockdown did not affect CRC cells proliferation.

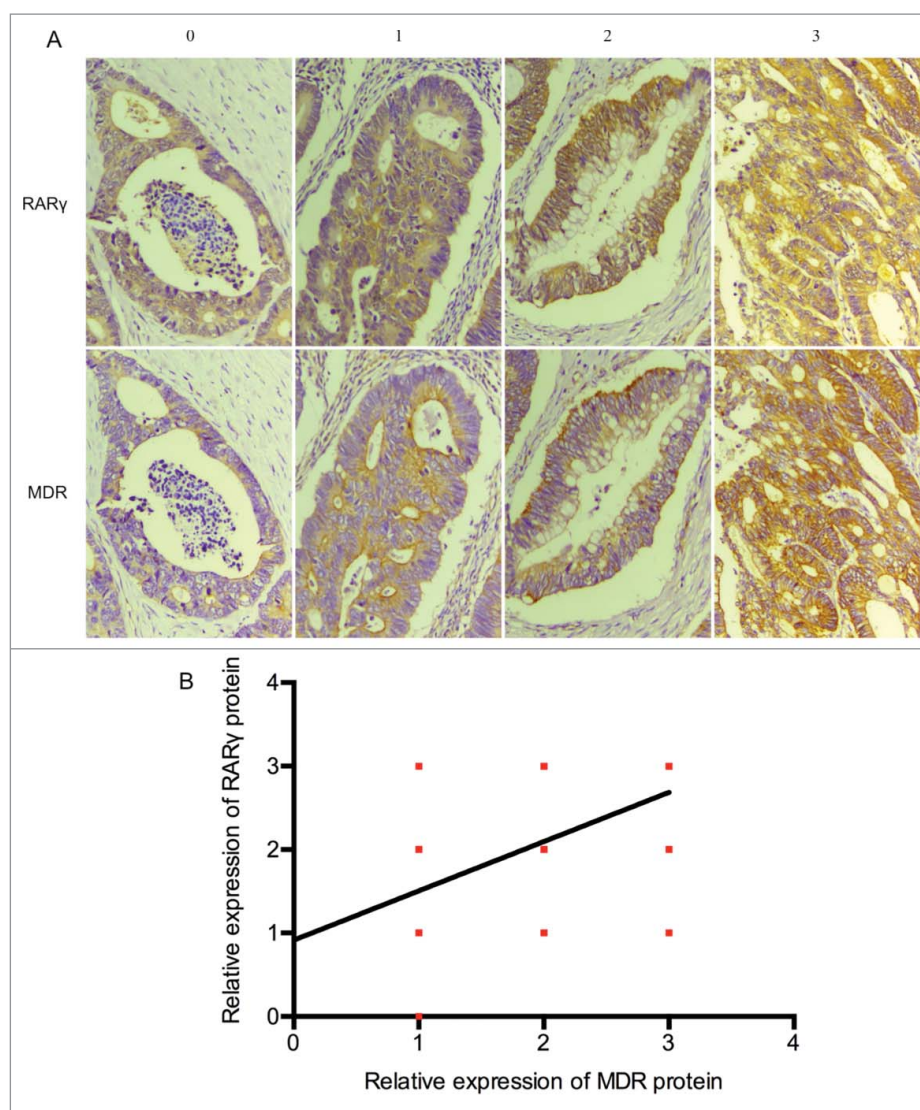


Figure 5. High RAR γ are correlated with the expression of MDR1 in human CRC tissues. (A) Representative immunostaining for RAR γ and MDR1 shown for 4 patient samples. (B) Correlations between RAR γ and MDR1 protein levels in 90 tumor tissues measured.

The most significant finding of our study is that lentiviral vectors carrying shRAR γ -mediated RAR γ silencing effectively sensitized CRC cells to chemotherapeutics, as reflected by a strong inhibition of MDR1 rather than MRP1, MRP2 or MRP3, which is in agreement with our previous studies.¹¹ The poor prognosis of CRC patients is largely due to development of chemoresistance.²⁴ Resistance to chemotherapeutic drugs is a major obstacle in the treatment of cancers, and such resistance has been related to the expression of multidrug-efflux transporters.²⁵ One of the best characterized mechanisms of MDR is overexpression of drug efflux transporters including ATP-binding cassette (ABC) drug transporter protein MDR1, and multidrug resistance-associated protein MRP1, MRP2 and MRP3, which decreases drug uptake and increases drug efflux in cancer cells.^{14,26} Previous studies showed that MDR1 serves as an important MDR protein of CRC.^{27,28} Of particular interest, the molecular mechanism of the way regulating MDR1 in CRC is still unclear.

The results from the present study demonstrated that Wnt/ β -catenin pathway was involved in the RAR γ -induced

upregulation of MDR1 in CRC. The aberrant activation of Wnt/ β -catenin signaling pathway is vital the development of cancers, including CRC.²⁹⁻³¹ Importantly, RAR γ was required for the transduction of Wnt/ β -catenin signaling and the activation of MDR1 in CRC cells, suggesting that RAR γ mediates the chemoresistance of CRC. What's more, RAR γ expression in CRC tissues is positively correlated with expression of MDR1.

In conclusion, our study has for the first time demonstrated RAR γ upregulates MDR1 expression via Wnt/ β -catenin pathway and mediating MDR of CRC cells. RAR γ could represent a potential therapeutic target for chemoresistant CRC patients.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Funding

This work was supported by Grants from the National Nature Science Foundation of China Grant Nos. 81572394 and 81402309; and

the National Science Foundation for Fostering Talents in Basic Research of the National Natural Science Foundation of China Grant No. J1310027.

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