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Original Article

RNA-binding protein RBM38: acting as a tumor suppressor in colorectal cancer

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Abstract: Background: As a member of RNA recognition motif (RRM) family of RNA binding proteins (RBPs), RNA binding motif protein 38 (RBM38) can regulate expression of diverse targets by mRNA stability and played an important role in cancer development, mostly acting as an oncogene in many human tumors. However, its role in human colorectal cancer (CRC) is controversy and needs further clinical and experimental confirmation. Objective: The aim of this study was to explore the expression pattern and biological function of RBM38 in CRC. Method: We analyzed RBM38 mRNA and protein expression in 54 CRC tissues and marched adjacent non-cancerous tissue or in 90 CRC tissues and 23 adjacent non-cancerous tissues. A lent virus approach was used to confirm the biological function of RBM38 in CRC cell lines. Results: We observed that RBM38 was frequently silenced in CRC tissues compared to adjacent normal colorectal tissues. Overexpression of RBM38 significantly inhibited cell proliferation, colony formation, cell migration. Conclusions: Therefore, our findings indicated that RBM38 acts as tumor suppressor and a promising biomarker for the diagnosis or treatment of CRC.

Keywords: RBM38, colorectal cancer, tumor suppressor

Introduction

Colorectal cancer (CRC) is the third common cancer worldwide with more than 1.2 million new cases each year [1, 2]. The five-year survival rate for CRC is 65% [3]. An improved and detailed understanding of the mechanisms underlying CRC pathogenesis is very important for the diagnosis or treatment of CRC. RNA binding proteins (RBPs) have been reported to form a complex network with oncogene and tumor suppressors and have profound impacts on tumor development and progression [4].

RBPs contain one or more RNA-binding motifs, such as the RNA recognition motif (RRM), the human heterogeneous nuclear ribonucleoprotein (hnRNP) K homology motif, the RGG box and the double-stranded RNA binding domain (dsRBD) motif [6, 7]. They are master regulators of RNA biogenesis and metabolism, such as polyadenylation, RNA splicing, transport, stability, and translation, all of which are emerging as critical mechanisms for gene regulation in mammalian cells [4, 5]. Many genes regulated by RBPs are responsible for cell growth and proliferation. So the altered expression and dysfunction of RBPs might cause defects in cell physiology and lead to cancer development [4, 8].

RBM38, also called RNPC1, belongs to the RRM family of RBPs and is expressed as two isoforms, RBM38a with 239 amino acids and RBM38b with 121 amino acids, respectively [9]. Both RBM38a and RBM38b contain one RRM which shares a sequence similarity with those in Hu antigen R (HuR) and Musashi [10]. RBM38 is known to interact with its target mRNAs and regulate their expression via mRNA stability. It plays pivotal roles in regulating wide biological processes, ranging from cell proliferation, cell cycle arrest to cell myogenic differentiation [9]. It is capable of regulating biological characteristics, binds and stabilizes the mRNA of p21, p73 and HuR [10-12]. Recently, RBM38

is also found to bind and stabilize the mRNA of macrophage inhibitory cytokine-1 (MIC-1) [13], which facilitates RBM38-induced cell growth suppression. Additional mRNAs bound by RBM38 include p63, murine double minute-2 (MDM2) and p53 mRNAs [14, 15].

RBM38 is located on chromosome 20q13, frequently amplified in breast cancer [16, 17], prostate cancer [18], ovarian cancer [19], chronic lymphocytic leukemia [20], colon carcinoma [21], esophageal cancer [22], dog lymphomas [23], and colorectal cancer [24, 25]. It was originally recognized as oncogene. But recently, new evidences suggested RBM38 might act as a tumor suppressor. It is reported that RBM is a part of a negative feedback loop, which restricts E2F1 activity by limiting cell-cycle progression at the G1-S boundary [26]. In addition, RBM38 was reported to be a tumor-suppressor in breast cancer [17]. Ding *et al* [9] have observed that there may exist a correlation between decreased expression of the RBM38 gene and poor survival in the colorectal cancer by bioinformatics analyses. It was contradictory with the previous studies that the RBM38 was recognized as oncogene in CRC [21]. However, this controversy on the role of RBM38 in the CRC needs further clinical and experimental confirmation.

In this study, we examined RBM38 expressive level in clinic colorectal cancerous and adjacent normal colorectal specimens by RT-PCR and IHC analysis, and analyzed the association of RBM38 expression with clinic pathological characters. The result showed that the RBM38 expression was lower in the CRC tissues compared with adjacent normal CRC tissues. RBM38 mRNA expression was associated with pathological grade. Then, we studied the effect of RBM38 in colorectal cancer cell lines HCT-116 and DLD-1 on cell proliferation, migration, and invasion. Both the clinical data and experimental results suggested that RBM38 might act as a tumor-suppressor in CRC.

Methods and materials

Tissue samples

Fifty-four CRC tumors and matched normal tissues from adjacent regions were obtained from patients treated surgically for clinical stage I-III colorectal cancer (aged 34-82 years) in the First Affiliated Hospital of Nanjing Medical University from February 2006 to August 2009. Patients did not receive chemotherapy, radiotherapy or hormone therapy before surgery. Tumor and normal tissue samples had been confirmed as tumor tissues or normal tissues by histopathological examination of hematoxylin stained paraffin sections. Histopathological types were classified according to the World Health Organization (2003). TNM staging was defined according to the American Joint Committee on Cancer (AJCC) (the 6th version, 2002). All the cases were individually categorized by independent pathologists. All the samples' collection was approved by the ethics and research committee of the First Affiliated Hospital of Nanjing Medical University according to the ethical guidelines of the Declaration of Helsinki. All patients were informed that their surgical specimens would possibly be used for research purposes before surgery and provided their written informed consent for inclusion in the data analysis and manuscript publication.

Cell culture

The colorectal cancer cell lines (DLD-1, HCT116) were obtained from Prof Damin Gao in Institute of Biochemistry and Cell Biology, SIBS, CAS. DLD-1 and HCT116 were cultured in RPMI 1640 medium (GIBCO) supplemented with 10% fetal bovine serum the FBS to here, just like (FBS, GIBCO, Australia), 1% penicillin-streptomycin (GIBCO). All cells were cultured and maintained in an incubator with humidified 5% CO_2 at 37°C.

Western blot analysis

The stably transfected cells were lysed, and the protein was extracted with RIPA buffer (Beyotime, China) and quantified by BCA protein assay kit (Beyotime, China). Equal amounts of total proteins were separated on 12% SDS-polyacrylamide gels (PAGE) and transferred to polyvinylidene difluoride membranes (PVDF) (Millipore, USA), which were activated in methanol. After blocking in 5% nonfat milk at 37°C for 2 h, the membranes were incubated with primary antibodies at 4°C overnight. The primary antibodies used were anti-rabbit RBM38 (1:500; Santa Cruz), anti-mouse β-actin (1:1000; Santa Cruz). Washed the membranes three times with TBST, then incubated with secondary antibodies for 1 h. Band signals were detected using a chemiluminescence system (Bio-Rad, USA). The β-actin expression was used as a loading control for whole cell lysates.

Transfection

Lentivirus constructs were generated to overexpress and know down RNPC1a. The CRC cells were stably transfected with PGLV3-h1-GFP-puro vector (GenePharma, Shanghai, China) containing the RNPC1a knockdown (termed as shRNPC1a) and a scrambled sequence (termed as SCR). For RNPC1a overexpression, the CRC cells were transfected with pGLV5-h1-GFP-puro vector (GenePharma, Shanghai, China) and a negative control sequence (termed as NC). The CRC cells were seeded into 6-wells plates and infected with the retroviruses when the cells were at 30% confluence. 5 μg/ml polybrene was added into the 6-wells plates to enhance the infection efficiency. Then puromycin (3 μg/ml) was used to help select the stable pooled populations of CRC cells. Western blotting analysis and quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) were applied to determine the efficiency of transduction.

Colony formation assay

The stably transfected cells were seeded into 6-well plates (500 cells/well) and maintained in RMPI 1640 containing FBS for 10-15 days. The colonies were fixed in methanol and stained with crystal violet solution after washed by PBS twice, then dried at room temperature. The colonies were imaged and quantified, and all cell colonies contained 50 or more cells.

Cell proliferation assay

Cell proliferation assay was performed using CCK-8 kit (Dojindo, Japan) according to the manufacturer's instructions or protocols. For cell proliferation assay, 2,000 stably transfected cells per well were seeded into 96-well plate then incubated at 37°C in a 5% CO_2 humidified atmosphere. Then CCK-8 was used to determine cell viability for 5 days, 100 μl of spent medium was replaced with an equal volume of fresh medium containing 10% CCK8, and then cells continued to be incubated at 37°C for 4 h, and the absorbance was finally determined at 450 nm using a Microplate Reader (ESON).

Wound healing assay

The stably transfected cells lines were cultured in 6 well plates. After the cells had grown to a full confluent monolayer, the cell monolayer was carefully scraped using a sterile tip to create a wound (scratch) and washed twice with phosphate-buffered saline (PBS) to remove any debris and then the cells were incubated in serum-free RPMI 1640 medium for 24 hours. Photographs of the wound were taken immediately (0 h) and 24 h after scraping. Quantification of cell motility by measuring the distance between the invading fronts of cells in three random selected microscopic fields for each condition and time point (0, 24 h).

Cell migration and invasion assays

Cell migration and invasion were assayed by using a chamber of 6.5 mm in diameter, with 8 mm pore size (Millipore, Millipore, NY, USA) in accordance with the manufacturer's protocol. For the invasion assay, the upper surface of the membrane was coated with BD Matrigel (BD Biosciences, USA) at 37°C for 4 h, whereas for the migration assay, the top chamber was not coated with BD Matrigel.

Briefly, 2×10^4 exponentially growing cells in 200 μl serum-free medium were added to the upper chamber and 600 μl of medium containing 10% FBS was added to the lower chamber for culture. The cells were cultured in a humidified atmosphere for 24 h at 37°C, and 5% CO_2 after 24 h, the cells on the top surface of the membrane were removed with a cotton swab. Migrated cells adhering to the underside of the membrane were fixed with 100% methanol for 30 min and stained using 0.5% crystal violet (Sigma, USA) for 20 min. The permeating cells were counted under a phase contrast microscope (100×). (Olympus, Tokyo, Japan).

RNA extraction, reverse transcription and quantitative RT-PCR (qPCR)

Total RNA was extracted from tissue samples or cultured cells using Trizol reagent (TaKaRa, A-79061), and cDNA was synthesized using Primescript RT Reagent (TaKaRa, Dalian) following manufacturer's instructions. The corresponding cDNA was used for quantitative real-time PCR using SYBR green real-time Master Mix (TaKaRa, Dalian). β-actin, a constitutive expression gene, was used as a reference to obtain the relative fold change for targets using the comparative Ct method.

RBM38 forward, 5'-ACGCCTCGCTCAGGAAGTA-3-'; RBM38 reverse, 5'-GTCTTTGCAAGCCCTC-TCAG3-'; β-actin forward, 5'-GCTGTGCT AT-

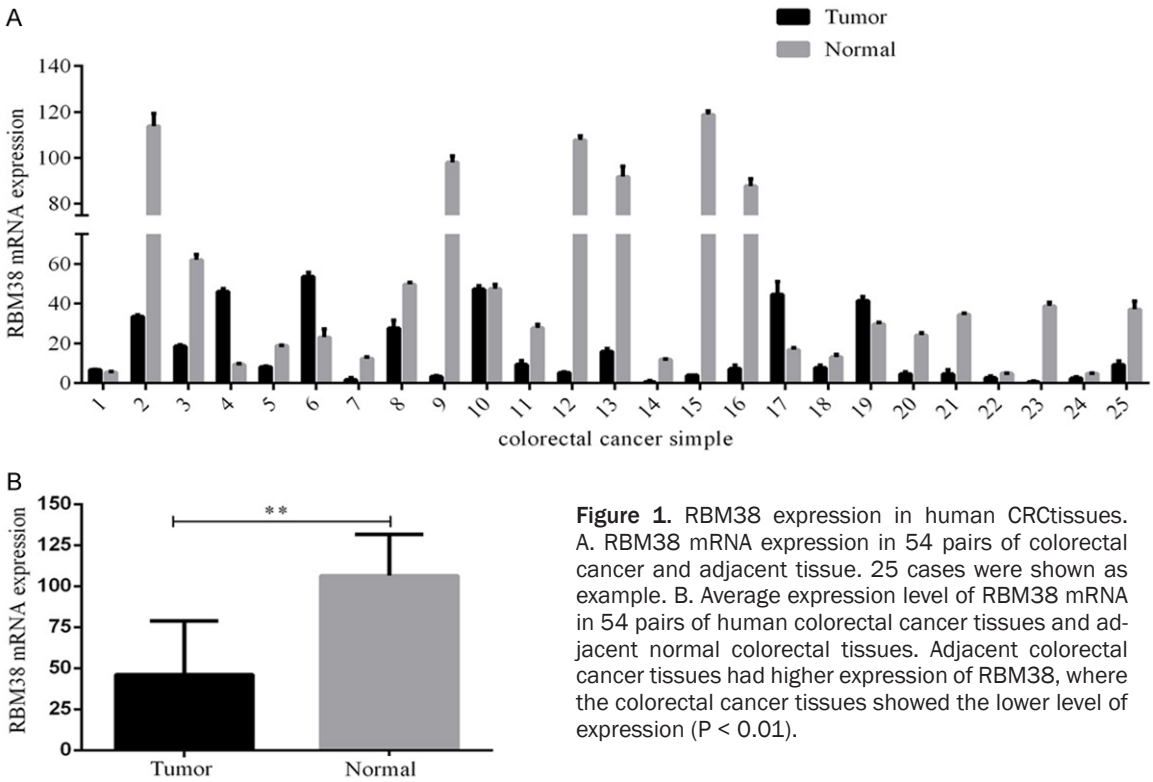

Figure 1. RBM38 expression in human CRCtissues. A. RBM38 mRNA expression in 54 pairs of colorectal cancer and adjacent tissue. 25 cases were shown as example. B. Average expression level of RBM38 mRNA in 54 pairs of human colorectal cancer tissues and adjacent normal colorectal tissues. Adjacent colorectal cancer tissues had higher expression of RBM38, where the colorectal cancer tissues showed the lower level of expression ($P < 0.01$).

Table 1. The association between RBM38 mRNA expression and clinicopathologic features of colorectal cancer

Clinicopathologic parameters	Number of case	RBM38 Low expression	RBM38 High expression	P
Age (years)				0.789
≤ 60	13	9	4	
> 60	40	24	16	
Tumor Size (cm)				0.329
< 5	31	21	10	
≥ 5	22	12	10	
TNM stage				0.199
I+II	17	13	4	
III+IV	36	21	15	
Lymph node metastasis				0.143
-	17	13	4	
+	36	20	16	
Grade				0.699
I	1	1	0	
II	30	19	11	
III	22	13	9	
Gender				0.748
Male	25	15	10	
Female	28	18	10	

CCCTGTACGC3-'; β-actin reverse, 5'-TGCCTCAGGGCAGCGGAACC3-'.

Immunohistochemistry

Specimens were fixed with neutral formalin, and 5 μm thick paraffin sections were made. Immunostaining was previously described using the avidin-biotin-peroxidase complex method (S-P Kit, Maixin, Fuzhou, China). Tissue sections were incubated with RBM38 antibody (1:150; Santa Cruz). Counterstaining with hematoxylin was performed and the sections were dehydrated in ethanol before mounting. Two independent pathologists examined all tumor slides. At least five random fields were examined each slide and 100 cells were observed per view. Staining of RBM38 was scored following a semi-quantitative scale by evaluating in representative tumor areas, the intensity, and percentage of cells. Cytoplasmic and membrane staining was considered as positive. The intensity was also scored as 0 (none), 1 (weak), and 2 (strong). Percentage scores were designated as 1 (1-25%), 2 (26-50%), 3 (51-

Table 2. RBM38 immunohistochemistry staining of colorectal cancer

Clinicopathological characteristics	RMB38 expression			*P*
	No. of cases	Low (%)	High (%)	
Gender				0.834
Male	38	18 (47.37)	20 (52.63)	
Female	52	26 (50.00)	26 (50.00)	
Pathological grade				0.017
I	8	2 (25.00)	6 (75.00)	
II	76	36 (47.37)	40 (52.63)	
III	6	6 (100)	0 (0)	
Age				0.836
≤ 60	42	20 (47.62)	22 (52.38)	
> 60	48	24 (50.00)	24 (50.00)	
TNM stage				0.376
I	2	0 (0)	2 (100)	
II	24	12 (50.00)	12 (50.00)	
III	64	32 (50.00)	32 (50.00)	
Lymph node metastasis				0.137
-	54	30 (55.56)	24 (44.44)	
+	36	14 (38.89)	22 (61.11)	
Tissue type				0.000
Cancer	90	44 (48.89)	46 (51.11)	
Non-cancer	23	2 (8.70)	21 (91.30)	

75%), and 4 (76-100%). The two scores were multiplied to get final score from 0 to 8. RBM38 was determined as low expression (score < 4); high expression (+) (score ≥ 4).

Statistical analysis

Statistical analysis performed with SPSS20 software and GraphPad Prism 6. All data are presented as the mean ± standard deviation. Differences between two groups were analyzed with the student t test. *P* value < 0.05 was considered statistically significant. All experiments were repeated more than three times, and each experiment was performed in triplicate.

Results

RBM38 mRNA expression was down-regulated in CRC tissue

To determine RBM38 expression in CRC tissues, we use qRT-PCR to analyze mRNA RBM38 in 54 pairs of CRC tissues. Of the 54 paired samples, 39 (72.2%) showed significantly lower RBM38 mRNA expression in the CRC tissue compared with the adjacent tissue, indicating that RBM38 might be a potential tumor suppressor in human CRC. Partial data was showed in **Figure 1A** (*P* = 0.003). Mean of RBM38 level in tumors or tumor-adjacent normal tissue was 46.03, 106.45, respectively (**Figure 1B**), which suggested that lower expression of RBM38 were common in colorectal cancer tissues compared with tumor-adjacent normal tissue.

Table 1 displayed the association of RBM38 expression level and clinicopathological features of 53 CRC patients. The data demonstrated that there was no significant correlation between RBM38 mRNA expression and patient age, tumor size, TNM stage, Lymph node metastasis, grade or gender.

Immunohistochemical (IHC) staining of RBM38 in human CRC tissues

To confirm the expressive level of RBM38 in CRC, IHC analysis was performed to investigate the expression of RBM38 in 90 CRC tissues and 23 tumor-adjacent normal tissues. The correlation between RBM38 expression and clinicopathological features was analyzed (**Table 2**). The expression of RBM38 was significantly associated with pathological grade (*P* = 0.017) and tissue type (*P* = 0.000). There was no significant correlation between RNPC1 expression and gender, age, TNM stage, lymph node metastasis. IHC staining for RBM38 was extremely positive in 21 of 23 (91.30%) non-cancer tissues, positive in 46 of 90 (51.11%) CRC tissues. The representative images of RBM38 expression in CRC and tumor-adjacent normal tissues were showed in **Figure 2**.

Establishment of stable CRC cell lines HTC-116 and DLD-1 overexpressing or knockdown RBM38

Our results demonstrated that RBM38 was strongly down-regulated in CRC specimens. Furthermore, several studies have confirmed that RBM38 has tumor-suppressive effects in breast cancer cells [17]. So we established the

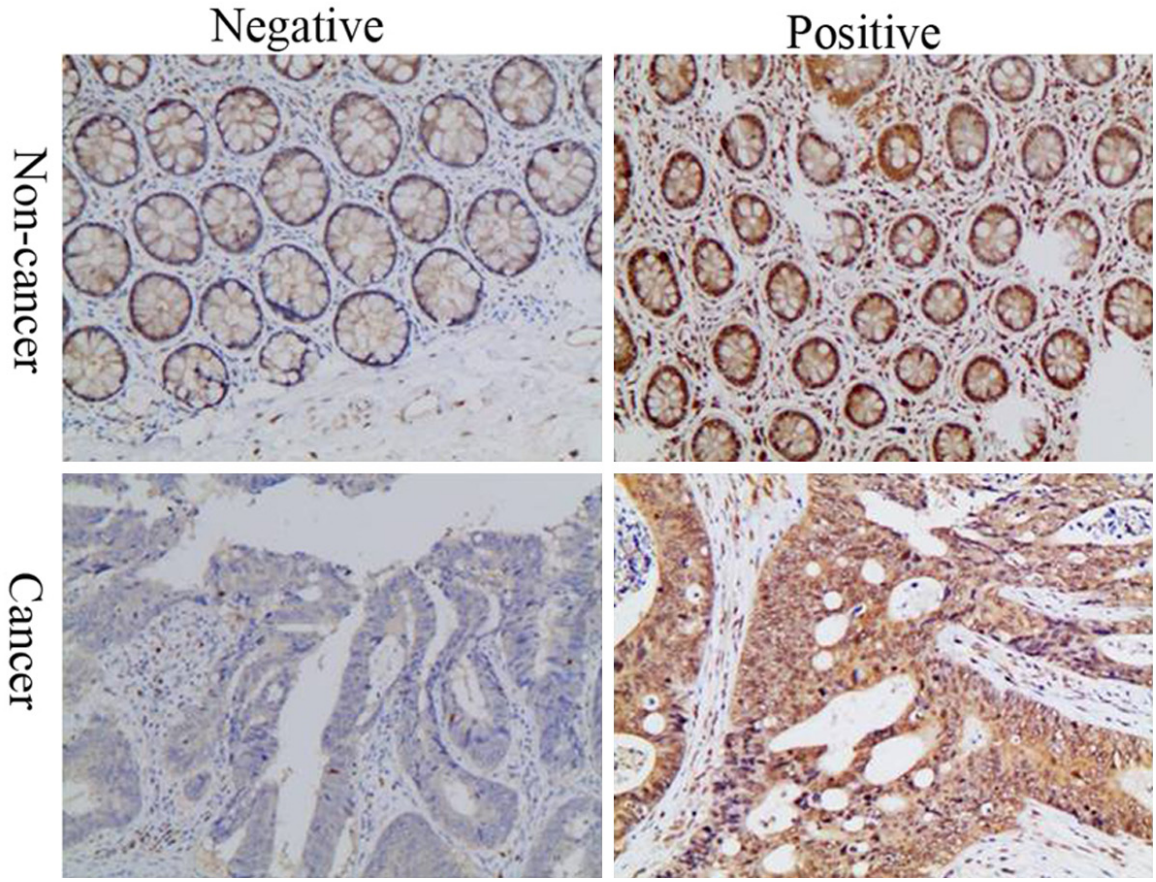

Figure 2. IHC staining of RBM38 in human CRC tissues.

stable CRC cell lines HTC-116 and DLD-1 over-expressing or knockdown RBM38. The over-expressed cell lines were named as HTC-116-RBM38 or DLD-1-RBM38, while the matched control cell lines were named as HTC-116-NC or DLD-1-NC, respectively. The silenced cell lines were named as HTC-116-shRBM38 or DLD-1-shRBM38, while the matched control cell lines were named as HTC-116-SCR or DLD-1-SCR, respectively. The protein and mRNA expression of RBM38 was confirmed by western blot (**Figure 3A**, **3E**) and qRT-PCR. (**Figure 3B**, **3F**).

RBM38 inhibited proliferation and growth in human CRC cells in vitro

The growth of the stable cell lines over 5 days was determined using cell counting kit (CCK-8) assay. As shown in **Figure 3C** and **3D**, RBM38 knockdown led to significantly increased cell proliferation ($P < 0.0001$, $P < 0.05$), while RBM38 over expression led to significantly decreased cell proliferation (**Figure 3G**, **3H**, $P < 0.0001$, $P < 0.05$).

Since anchorage-independent growth is strongly correlated with tumorigenicity. The colorectal cancer cell lines stably transfected were plated and incubated for 15-20 days. The ability of HCT116 or DLD-1 cell lines to form colonies was much more when RBM38 was knockdown (**Figure 4A**, both $P < 0.05$). The ability of HCT116 or DLD-1 cell lines to form colonies was much fewer when RBM38 was over expressed (**Figure 4B**, both $P < 0.05$).

RBM38 suppressed migratory and invasive potential of human CRC cells in vitro

The results of wound healing assay were shown in **Figure 5A** and **5B**. After 24 h, distance migrated of RBM38 knockdown increased by 49.5 mm (**Figure 5A**, $P < 0.01$) and RBM38 over expression decreased by 31.7 μm (**Figure 5B**, $P < 0.01$) compared to the control, respectively.

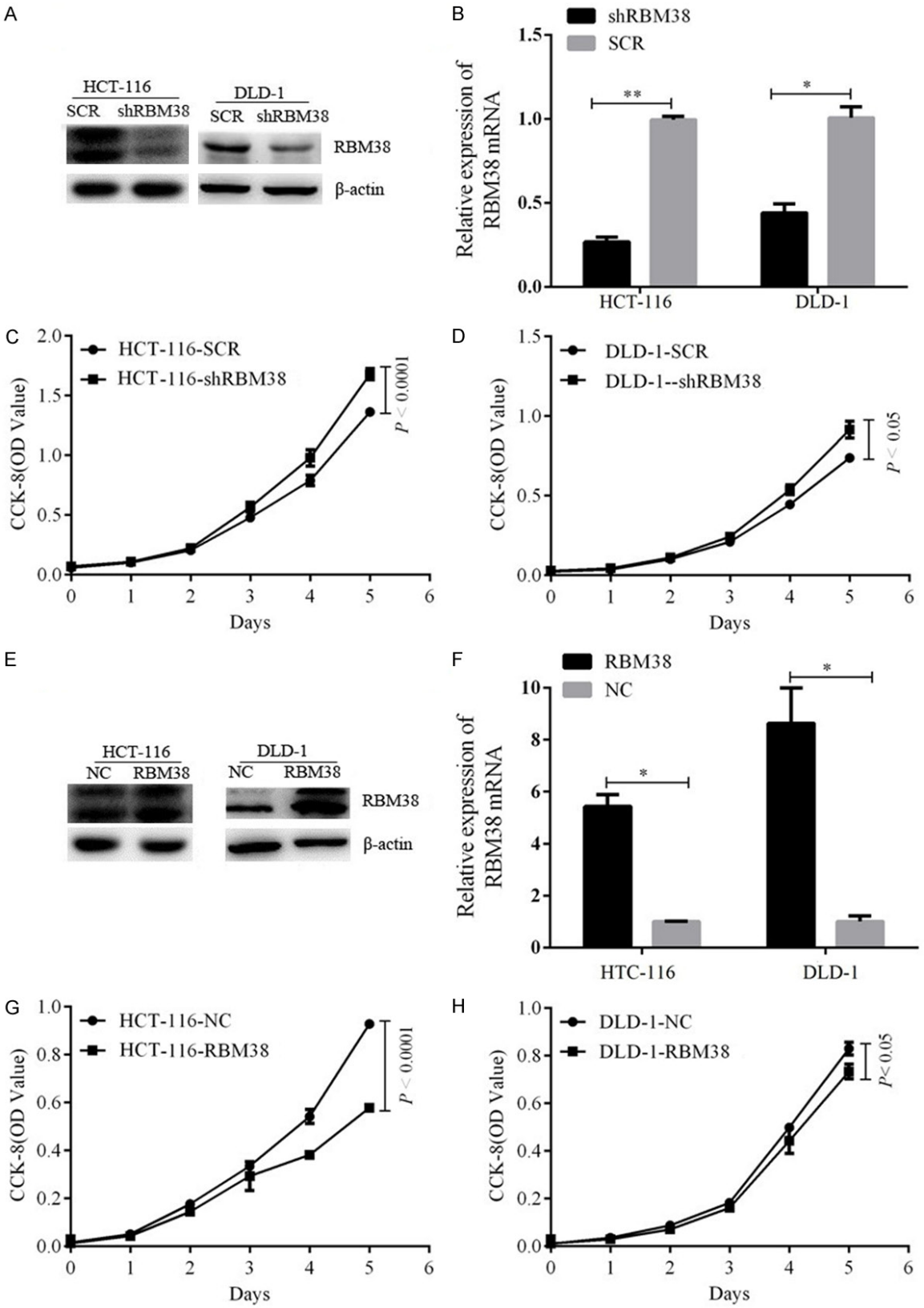

Figure 3. Effect of RBM38 on the proliferation and growth of CRC cell lines HCT-116 and DLD-1. (A). Western blot and (B) qRT-PCR were used to verify the efficiency of RBM38 knockdown. (C, D) The growth of cells over 5 days was measured using CCK-8 assays. HCT-116-shRBM38 and DLD-1-shRBM38 were significantly increased compared with control cells, respectively. Data were means of three separate experiments mean ± SEM. (E) Western blot and

(F) qRT-PCR were used to verify the efficiency of RBM38-overexpression. (G, H) HCT-116-RBM38 and DLD-1-RBM38 were significantly decreased compared with control cells, respectively. Data were means of three separate experiments mean ± SEM, *P < 0.05, **P < 0.01.

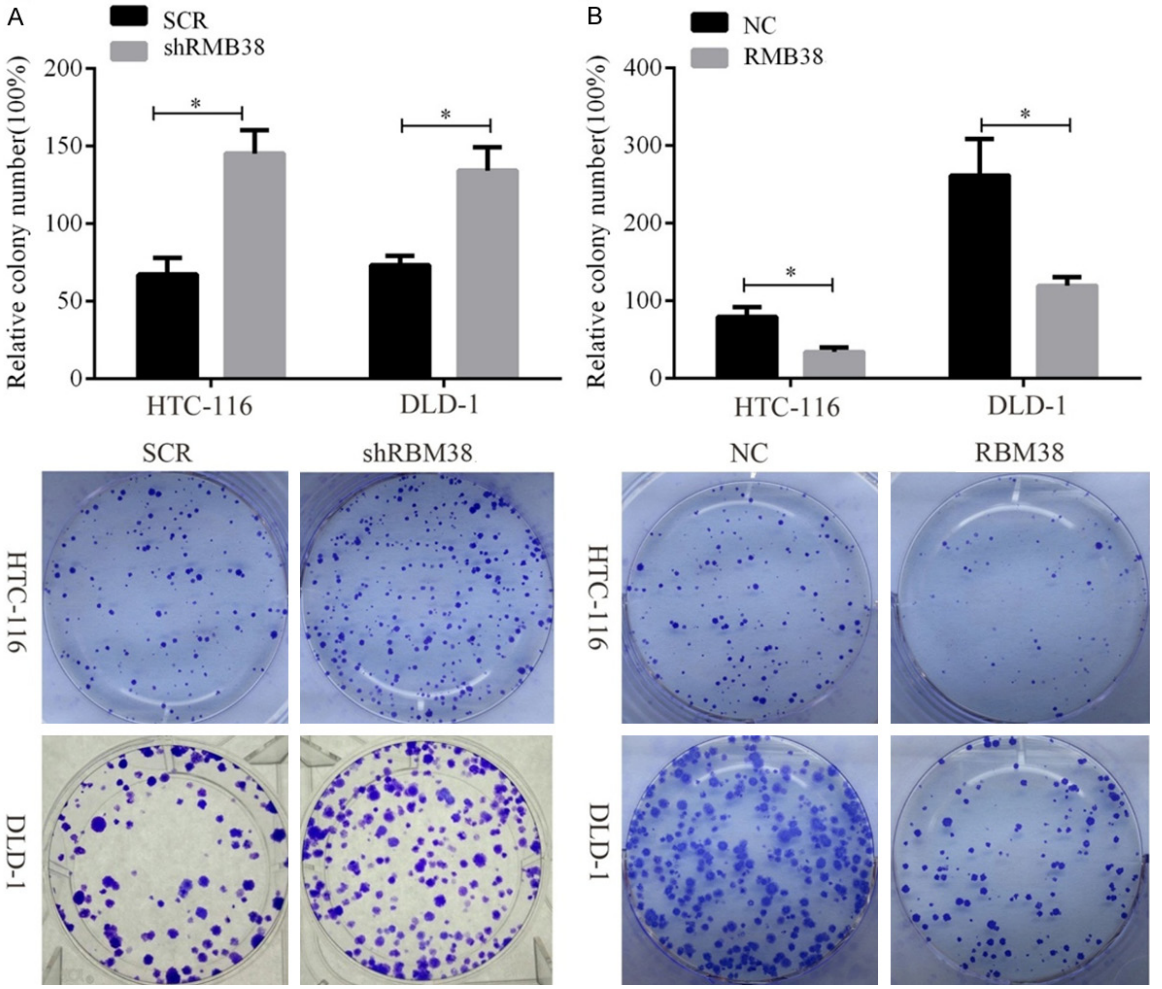

Figure 4. RBM38 suppressed the growth of CRC cells. A. The growth of cells over 15 days was measured using colony formation assays. The number and size of HCT-116-shRBM38 and DLD-1-shRBM38 was significantly increased compared to control cells (SCR), respectively. Data were means of three separate experiments mean ± SEM, P < 0.05. B. Clone formation of RBM38 overexpression arbitrarily set at 100% in control cells (NC). The number and size of HCT-116-RBM38 and DLD-1-RBM38 was significantly decreased compared with control cells, respectively. Data were means of three separate experiments mean ± SEM, P < 0.05. Colonies > 50 mm were counted. Anchorage-dependent growth assays were shown at the bottom. Data were means of three separate experiments mean ± SEM, *P < 0.05.

We conducted three-dimensional cell migration assay using transwell chambers and invasion assay with matrigel-precoated transwell chambers. We found that RBM38 knockdown exhibited significantly increase ability of migration and invasion (**Figure 6A**, **6B**, both $P < 0.05$). We found that RBM38 over expression exhibited significantly decrease ability of migration and invasion (**Figure 6C**, **6D**, $P < 0.05$, $P < 0.01$).

Discussion

Colorectal cancer (CRC) ranks the third among the estimated cancer cases and cancer related mortalities. Early detection and efficient therapy of CRC remains a major health challenge. Therefore, there is a need to identify novel tumor markers for early diagnosis and treatment of CRC [27]. RBPs have been reported to be involved in the cancer development. As a

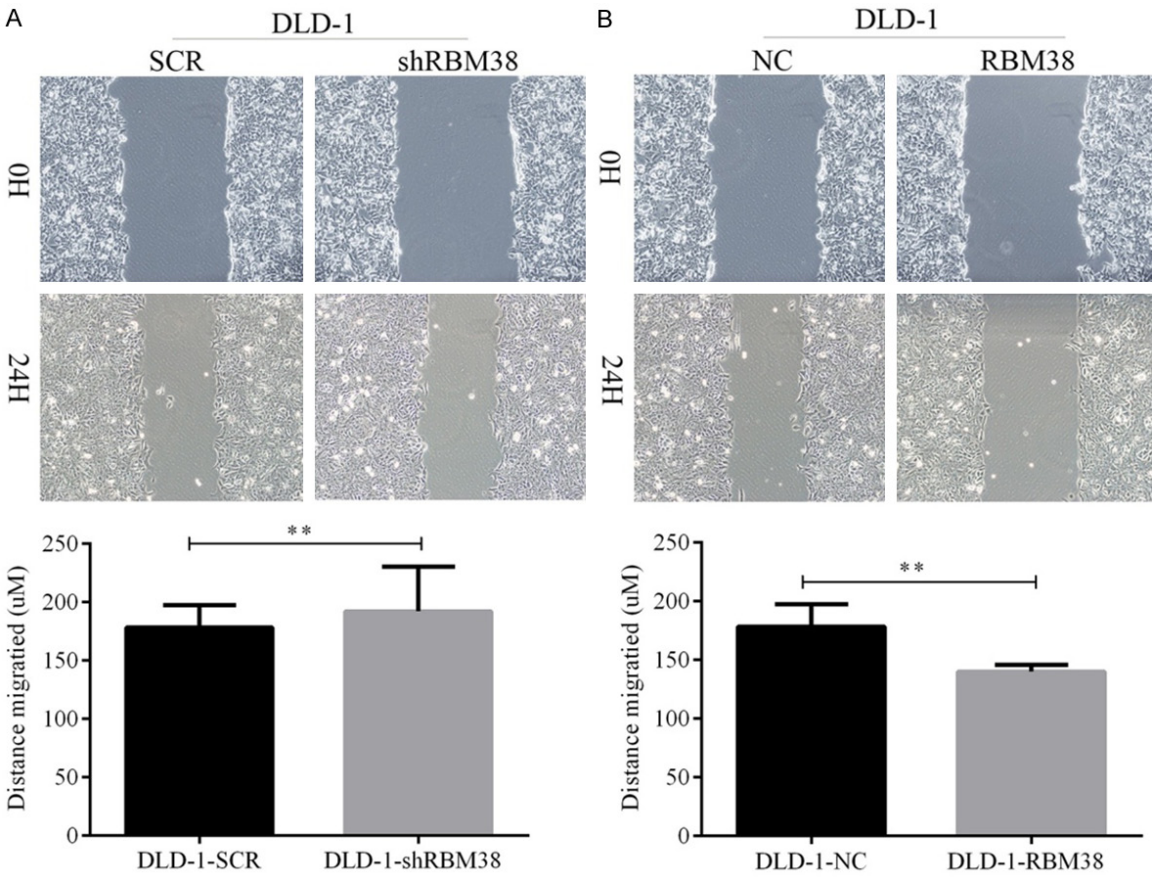

Figure 5. RBM38 significantly decreased migratory potential of CRC cells. Wound healing assay images of wound repair were taken at 0, 24 h after wound. The distance of wound closure is shown by area at 24 h. Representative photographs (upper) and quantification (lower) are shown.

member of RRM family of RBPs, RBM38 was proved to plays pivotal roles in regulating cell proliferation, cell cycle arrest, cell myogenic differentiation and so on [10, 28]. To date, increasing evidences have demonstrated that RBM38 play a vital role in tumor progress. RBM38 was originally recognized as an oncogene in CRC, for it was frequently found to be amplified in CRC tissues. However, Ding et al [9] have observed that there is maybe an exist correlation between decreased expression of the RBM38 gene and poor survival in the colorectal cancer by bioinformatic analyses. In the present study, we confirmed that RBM38 indeed acted as a tumor suppressor in CRC combined by clinical observations and experimental studies.

We observed the mRNA expression of RBM38 in 54 paired CRC tissues and adjacent normal tissues. Meanwhile, we analyzed the RBM38 expression with the IHC analysis in 90 CRC tissues and 23 tumor-adjacent normal tissues. It was shown that RBM38 expression is down-regulated in CRC tissues (**Figure 1A** and **1B**). The expression of RBM38 was not correlated with the patient's age and gender (**Table 1**). The main reason may be that surgical resection is restricted to early and local colorectal cancers. However, this study only examined one set of cancer samples from a single clinical center. In the future, we hope to increase the simple size and draw patients from multiple clinical research centers to verify these results. To further assess the role of RBM38 in CRC more in detail, we established the stable CRC cell lines overexpressing or knockdown RBM38. Then colony formation and CCK-8 assay were performed to explore the change of the proliferation in CRC cell lines. The results were consistent with the clinical observation, that overexpression of RBM38 could suppress CRC cell proliferation. The wound healing assay and cell migration and invasion assays indicated that

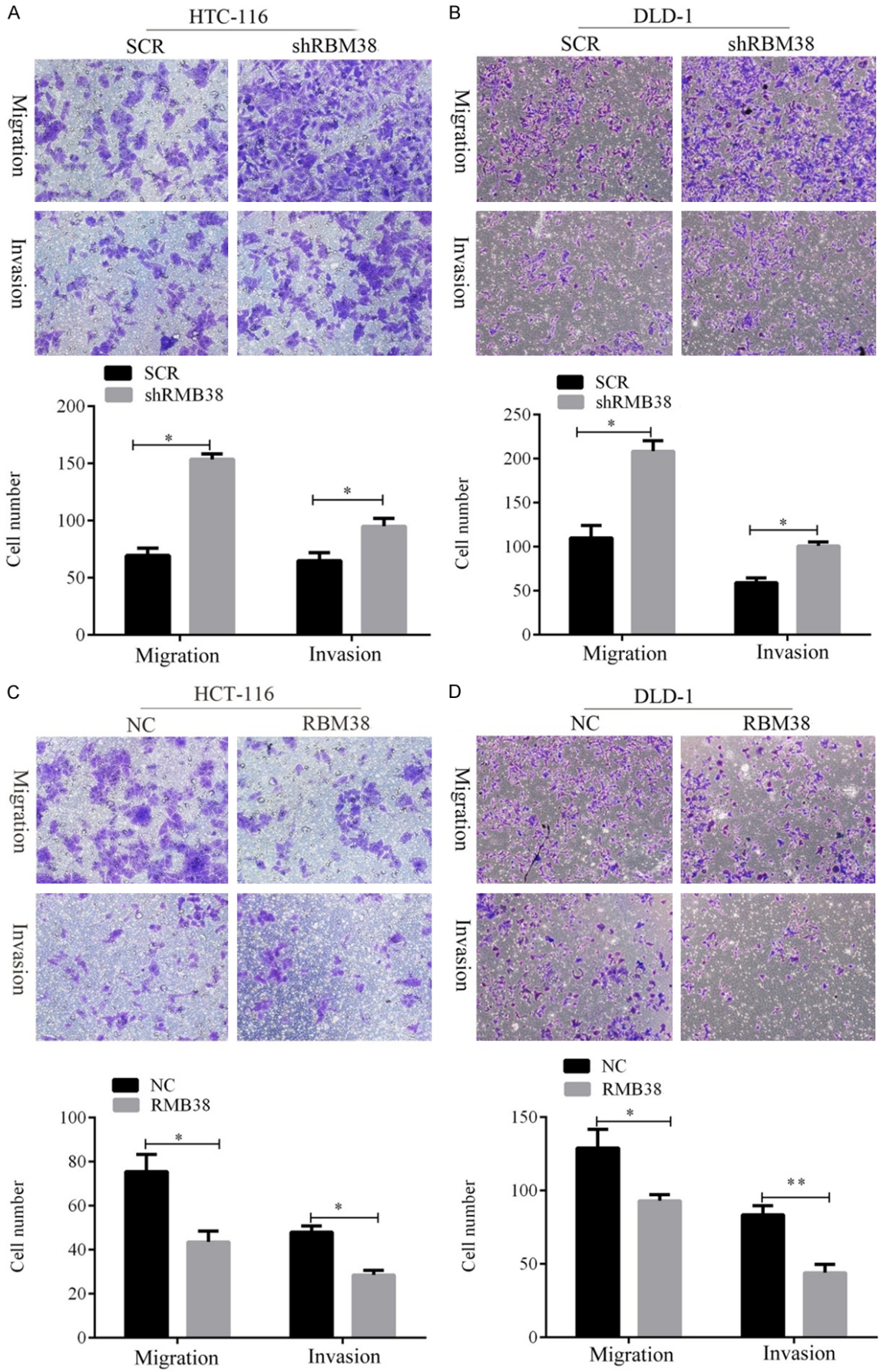
A
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Figure 6. RBM38 significantly decreased migratory and invasive potential of CRC cells. A, B. Transwell migration assay and Matrigel invasion assay of HTC-116-SCR, HTC-116-shRBM38, DLD-1-SCR, DLD-1-shRBM38 cells. C, D. Transwell migration assay and Matrigel invasion assay of HTC-116-NC, HTC-116-RBM38, DLD-1-NC, DLD-1-RBM38 cells. Representative photographs (upper) and quantification (lower) are shown. Columns: average of three independent experiments, *$p < 0.05$, **$p < 0.01$.

RBM38 can suppress migration and invasion of CRC cells in vitro. These clinical data and experimental results indicated that the RBM38 may play a tumor suppressor role in CRC. It was consistent with the bioinformatics analyses by Ding *et al* [9].

The mechanisms underlying the role of RBM38 in the process of tumors may be involving the mRNA stabilizing of oncogenes or anti-oncogenes, such as p53, p63 [14], MDM2 [15], p73 [11], HuR [29] and p21 [10, 12]. RBM38, a P53 target, is also a critical regulator of P53 translation. P53 gene is found to be deleted or mutated in 65% to 85% of CRC patients [30]. Recently studies have identified a novel p53-RBM38 auto-regulatory loop and suggest that RBM38 plays a role in tumorigenesis by repressing p53 translation [23]. Former study found that RBM38 and wtp53 were negative feedback loop. RBM38 overexpression inhibited mutp53 in colon cancer. Zhang *et al* [23] showed that the level of p53 protein was markedly increased by total RNPC1 or RNPC1a knockdown CRC cell HCT116. And in the CRC cell SW480 the mutant p53 protein was also significantly increased by RNPC1 knockdown. RNPC1a inhibited p53 translation in HCT116 and SW480 cells. The detailed mechanism by how RBM38 affects CRC development remained unclear and need further investigation.

Together, our findings demonstrate that RBM38 is low-expressed in human CRC tissues. In addition, RBM38 suppress the proliferation, colonies formation invasion and migration of CRC cells in vitro. We strongly believe that RBM38 may serve as a tumor suppressor in CRC.

Acknowledgements

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Disclosure of conflict of interest

None.

Authors' contribution

J.F.W and Y.B.D have contributed to the conception and design of the study. G.C, C.M.J and N.Y performed the experimental study and the analysis and interpretation of data. L.M performed the data analysis. All authors read and approved the final version of manuscript.

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