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ORIGINAL ARTICLE

RNF38 enhances 5-Fluorouracil resistance in colorectal cancer by activating the Wnt pathway

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Summary

Purpose: *Colorectal cancer (CRC) is a frequent fatal cancer worldwide. 5-Fluorouracil (5-FU) is extensively used in chemotherapy. Its drug resistance, however, should be well concerned. Ring finger proteins (RNF) are vital regulators involved in CRC development. In this article, HCT116R cells were first established. The roles of RNF38 and Wnt signaling in 5-FU-resistant CRC were further illustrated. Our study provides novel evidence for improving 5-FU chemotherapy outcome in CRC patients.*

Methods: *The phenotype of established HCT116R cells was first examined. Next, the regulatory effect of RNF38 on 5-FU-resistance in CRC was mainly explored. Nude mice bearing CRC were treated with 5-FU and in vivo overexpression of RNF38.*

Results: *5-FU-resistant HCT-116 cells (HCT116R) were first established. 5-FU treatment markedly decreased survival and induced apoptosis in HCT-116 cells. P53 was downregulated in HCT116R cells. Through microarray analysis, RNF38 was found upregulated in HCT116R cells than that of parental cells.*

Conclusions: *Overexpression of RNF38 enhanced 5-FU resistance in CRC. Furthermore, Wnt signaling was activated by RNF38 and involved in 5-FU resistance in CRC.*

Key words: *CRC, RNF38, 5-FU, chemotherapy, resistance*

Introduction

Colorectal cancer (CRC) is the third most frequent cancer worldwide with high mortality [1]. 5-FU is nowadays widely applied in CRC chemotherapy. Its drug resistance, however, greatly limits the therapeutic efficacy [2]. The anti-cancer effect of 5-FU is mainly due to its ability of DNA damage, leading to cell growth suppression and apoptosis [3-5]. Clinically, 5-FU resistance influences life quality and increases mortality in cancer patients [6]. Underlying mechanisms of 5-FU resistance help improve prognosis in CRC patients.

Checkpoint network is activated following chemotherapy-induced DNA damage. It is reported that many vital factors and genes are involved in 5-FU resistance, including ataxia-telangiectasia and Rad3-related (ATR), p53 and checkpoint kinase 1 (CHK1). P53, as a tumor suppressor gene, is activated by 5-FU and further arrests cell cycle [7]. Mutation of p53 is observed in over 60% of CRC patients. Mutant p53 is a vital indicator determining 5-FU sensitivity in cancer patients [8]. Interestingly, cancer cells carrying wild type (WT) p53 are prone to drug resistance [9]. CHK1, which can be activated by ATR, is able to activate p53 [10]. These abovementioned genes are of significance in regulating chemotherapy resistance.

Ring finger proteins (RNF) belong to the Zinc finger protein family, most of which possess the activity of atypical E3 ubiquitin ligase to degrade proteins [11]. To date, several RNF proteins have been identified to be involved in cancer progression. RNF6 is proven to deteriorate CRC by activating the

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Wnt signaling [12]. RNF31 mediates breast cancer development by degrading p53 [11]. Through activating NF-κb, RNF183 stimulates proliferative potential of CRC [13]. Biological functions of RNF in 5-FU resistant CRC, however, remain unclear.

In this article, HCT116R cells were first established. The roles of RNF38 and Wnt signaling in 5-FU resistant CRC were further illustrated. Our study provides novel evidence for improving 5-FU chemotherapy outcome in CRC patients.

Methods

Establishment of 5-FU resistant CRC cell line

HCT-116 cells were induced with 0, 0.01, 0.1, 0.5, 2, and 10 mg/mL 5-FU for 6 months. Obtained HCT116R cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Rockville, MD, USA) containing 10 mg/mL 5-FU, 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) and penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA) in a humidified 5% CO_2 incubator at 37°C.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Cellular RNA isolated using TRIzol reagent (Qiagen, Hilden, Germany) was reversely transcribed by PrimeScriptTM RT reagent Kit (TaKaRa, Tokyo, Japan). RT-PCR was conducted using SYBR Q-PCR Super Mix-UDG (Invitrogen, Carlsbad, CA, USA).

Microarray analysis

10 mg total RNA were collected and subjected to synthesis of first-strand and second-strand complementary DNA (cDNA). After removal of double-stranded cDNA and transcription, cDNA was purified and fragmented into small sequences. 20 mg biotinylated cDNA were hybridized for Affymetrix 3'IVT Microarray analysis (Affymetrix, Santa Clara, CA, USA) at 45°C overnight. Subsequently, the microarray was scanned by phycoerythrin-conjugated streptavidin by a fluidics station (GeneChip Fluidics Station 450, Santa Clara, CA, USA).

Cell survival determination

The number of viable cells in 12-well plates following corresponding treatment was examined by the Countstar Automated Cell Counter (Shanghai, China). Survival rate was calculated as the percentage of survival cell number to total ones.

TUNEL assay

Cells were inoculated in 6-well plates covered by acid-treated slides. 24 h later, cover slides were washed and reacted in 4% formaldehyde for 60 min. Cells were penetrated in 1% Triton X-100 for 5 min and 3% H_2O_2 for 10 min. After washing with ice-cold phosphate buffered saline (PBS), cells were labeled with Terminal deoxynucleotidyl transferase (TdT) marker at 37°C for 1 h. Finally, cells were counterstained with dyeing buffer and 4',6-diamidino-2-phenylindole (DAPI), and captured.

Transfection

RNF38 siRNA, pcDNA-RNF38 or negative control were transfected using Oligofectamine reagent (Invitrogen, Carlsbad, CA, USA). Transfected cells for 48 h were harvested.

Western blot

Cellular protein extracted using cytobuster (Merck-Millipore, Billerica, MA, USA) was quantified by Bio-Rad protein assay system (Bio-Rad, Hercules, CA, USA). 10 μg protein sample were separated by 12% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and transferred on polyvinylidene difluoride membranes. After blockage in 5% bovine serum albumin (BSA), membranes were reacted with primary [rabbit anti-p53 (ab31333, Abcam, Cambridge, MA, USA), mouse anti-Wnt (ab169175, Abcam, Cambridge, MA, USA), rabbit anti-RNF38 (ab121487, Abcam, Cambridge, MA, USA) and rabbit anti-GAPDH (ab70699, Abcam, Cambridge, MA, USA)] and secondary antibodies. Electrochemiluminescence (ECL) Western blot reagents (Pierce Bio., Rockford, IL, USA) were used for band exposure.

Xenograft model

4-week-old nude mice were administered with HCT116 cells with corresponding treatment at the right side of the back. 5-FU was given intraperitoneally twice a week. Tumor size was weekly recorded. Four weeks later, mice were sacrificed for collecting CRC tissues.

Statistics

GraphPad Prism Software Version 7.0 (GraphPad Software Inc., La Jolla, CA, USA) was used for statistical processing. Differences between groups were compared by the Student's t-test. Data were expressed as mean ± SD. $P<0.05$ was considered as statistically significant.

Results

Identification of 5-FU resistant CRC cells

The phenotype of established HCT116R cells was first examined. Compared with parental cells, HCT116R cells were resistant to 5-FU induction (Figure 1A). Through observing cell morphology, HCT116 cells were almost all killed following 10 mg/mL 5-FU induction, while HCT116R cells presented normal shapes (Figure 1B). Apoptosis was pronounced in 5-FU-treated HCT116 cells. On the contrary, HCT116R cells were resistant to 5-FU-induced apoptosis (Figure 1C). Protein level of p53, an indicator of 5-FU-resistance, was markedly downregulated in HCT116R cells (Figure 1D). Our results confirmed the successful establishment of HCT116R cell line.

RNF38 was upregulated in HCT116R cells

Through microarray analysis, RNF38 was established as the most pronounced one to be up-

regulated in HCT116R cells (Figure 2A). We further validated that RNF38 was highly upregulated in HCT116R cells compared with parental cells (Figure 2B).

Overexpression of RNF38 enhanced 5-FU resistance in CRC

Next, the regulatory effect of RNF38 on 5-FU-resistance in CRC was mainly explored. Overex-

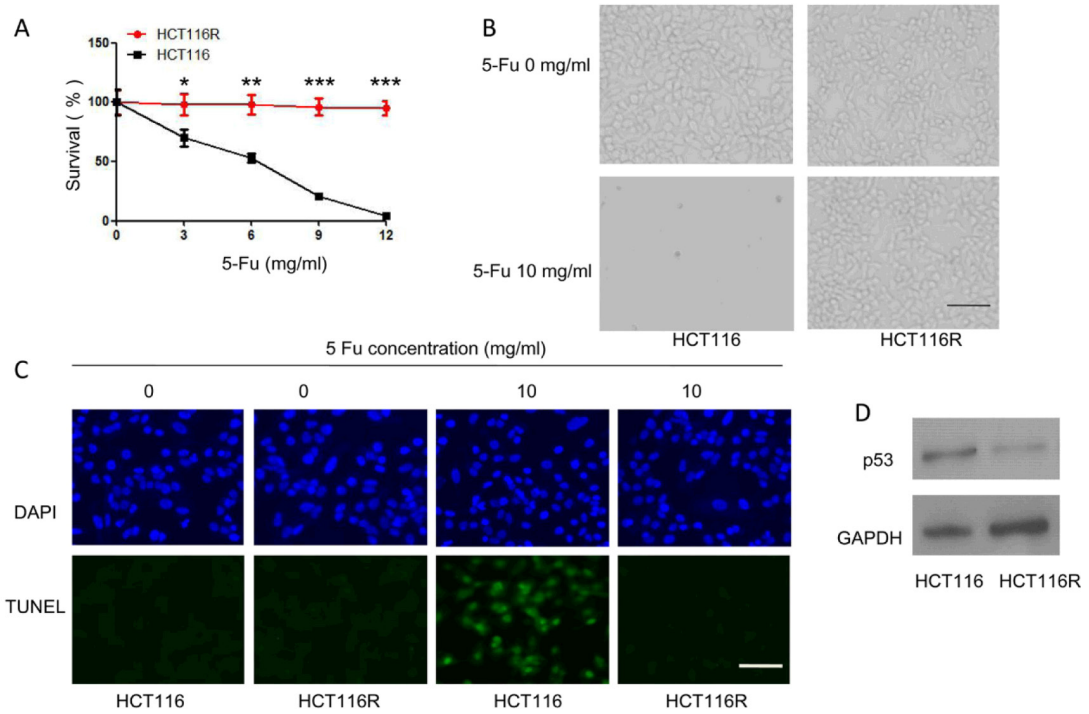

Figure 1. Identification of 5-FU-resistant CRC cells. **A:** Survival rate of HCT116 cells and HCT116R cells. **B:** Morphology of HCT116 and HCT116R cells either treated with 10 mg/mL 5-FU or not. Magnification 400×. Scale bar=50 μm. **C:** TUNEL assay showed apoptosis in HCT116 and HCT116R cells either treated with 10 mg/mL 5-FU or not. Magnification 200×. Scale bar=100 μm. **D:** Protein level of p53 in HCT116 and HCT116R cells. *p<0.05; **p<0.01; ***p<0.001.

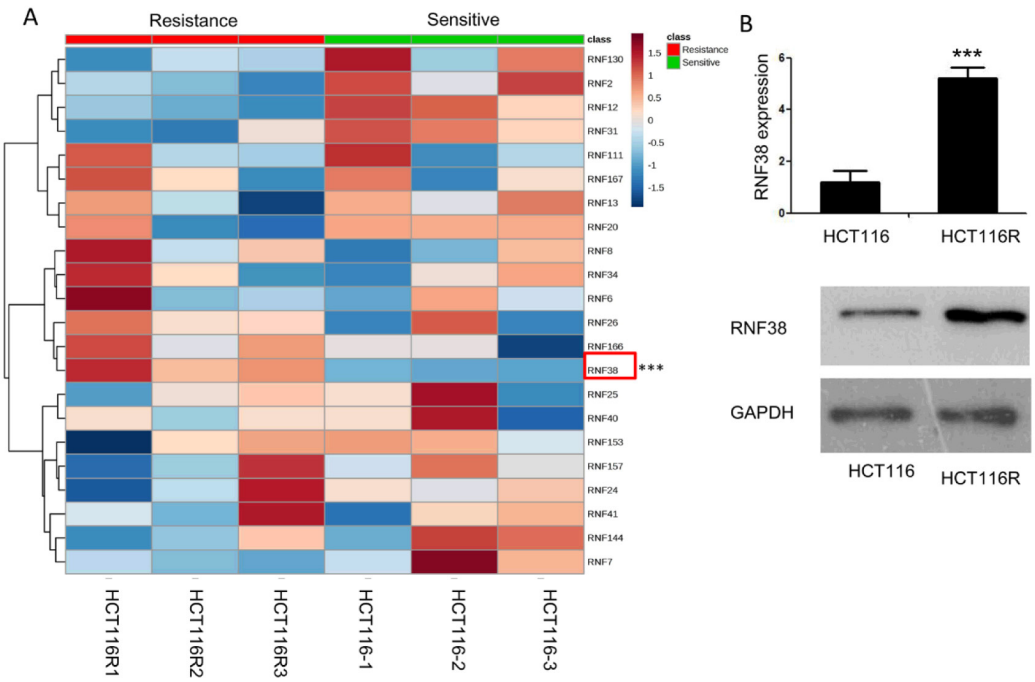

Figure 2. RNF38 was upregulated in HCT116R cells. **A:** Microarray analyses on differentially expressed RNFs in radioresistant or sensitive HCT116 cells. **B:** The mRNA and protein levels of RNF38 in HCT116 and HCT116R cells. ***p<0.001.

pression of RNF38 protected 5-FU-induced cell death and apoptosis in HCT116 cells (Figure 3A & 3B). In HCT116R cells, knockdown of RNF38 increased 5-FU sensitivity and stimulated apoptosis (Figure 3C & 3D). Moreover, the protein level of p53 was downregulated in HCT116 cells overexpressing RNF38 (Figure 3E). It is concluded that RNF38 could induce 5-FU resistance in CRC.

Nude mice bearing CRC were treated with 5-FU and *in vivo* overexpression of RNF38. Compared with those administered with untreated HCT116 cells, 5-FU treatment markedly reduced tumor size in nude mice bearing CRC. However, overexpression of RNF38 damaged the protective role of 5-FU in CRC growth, manifesting as a larger tumor size (Figure 3F).

Wnt signaling was involved in RNF38-mediated 5-FU resistance

Previous studies have reported the involvement of Wnt signaling in 5-FU resistant CRC cells [14-16]. Here, we demonstrated that knockdown of RNF38 downregulated Wnt in HCT116R cells (Figure 4A). Conversely, overexpression of RNF38 upregulated Wnt in HCT116 cells (Figure 4B). Silence of Wnt aggravated 5-FU-induced cell death in HCT116R cells and its parental cells (Figure 4C). Besides, knockdown of Wnt stimulated apoptosis in CRC (Figure 4D). P53 was also upregulated with Wnt knockdown in both RNF38 over-expressed HCT116 cells and HCT116R cells (Figure 4E). As a result, we believe that Wnt signaling is involved in RNF38-induced 5-FU resistance in CRC.

Inactivation of the Wnt signaling reduced 5-FU resistance in CRC in vivo

In vivo knockdown of Wnt in nude mice bearing RNF38-overexpressed CRC following 5-FU administration markedly reduced tumor size than those of controls. Similar findings were also found in mice administered HCT116 cells with knockdown

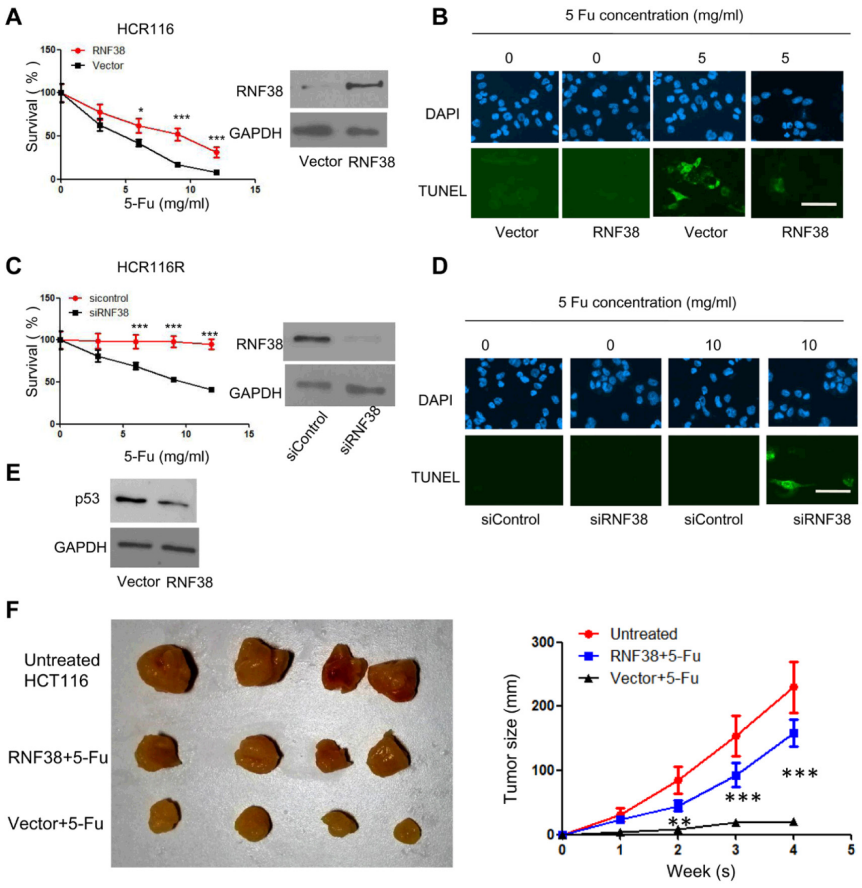

Figure 3. Overexpression of RNF38 enhanced 5-FU-resistance in CRC *in vitro*. **A:** Survival rate of HCT116 cells either with RNF38 overexpression or not. **B:** TUNEL assay showed the influence of overexpressed RNF38 on apoptosis in HCT116 cells treated with 10 mg/mL 5-FU or not. Magnification 200×. Scale bar=100 μm. **C:** Survival rate of HCT116R cells either with RNF38 knockdown or not. **D:** TUNEL assay showed the influence of silenced RNF38 on apoptosis in HCT116R cells treated with 10 mg/mL 5-FU or not. Magnification 200×. Scale bar=100 μm. **E:** Protein level of p53 in HCT116 cells either with RNF38 overexpression or not. **F:** Overexpression of RNF38 enhanced 5-FU resistance in CRC *in vivo*. Tumor size in nude mice administered with untreated HCT116 cells, or HCT116 cells either with RNF38 overexpression or not, followed by 5-FU administration. *p<0.05; **p<0.01; ***p<0.001.

of Wnt (Figure 4F). These results further indicated the critical function of the Wnt signaling in 5-FU resistant CRC regulated by RNF38.

Discussion

5-FU resistance is an obstacle during CRC treatment. Recent studies have shown the vital functions of RNFs in the development of different types of cancer [11-13]. For exploring the role of RNFs in 5-FU resistant CRC, we established HCT116R cells and conducted microarray analysis which showed that RNF38 was the mostly upregulated RNF member in resistant CRC. Our findings further demonstrated the role of RNF38 in enhancing both *in vitro* and *in vivo* 5-FU resistance in CRC.

The Wnt signaling is activated in cancer development, which is capable of regulating cancer

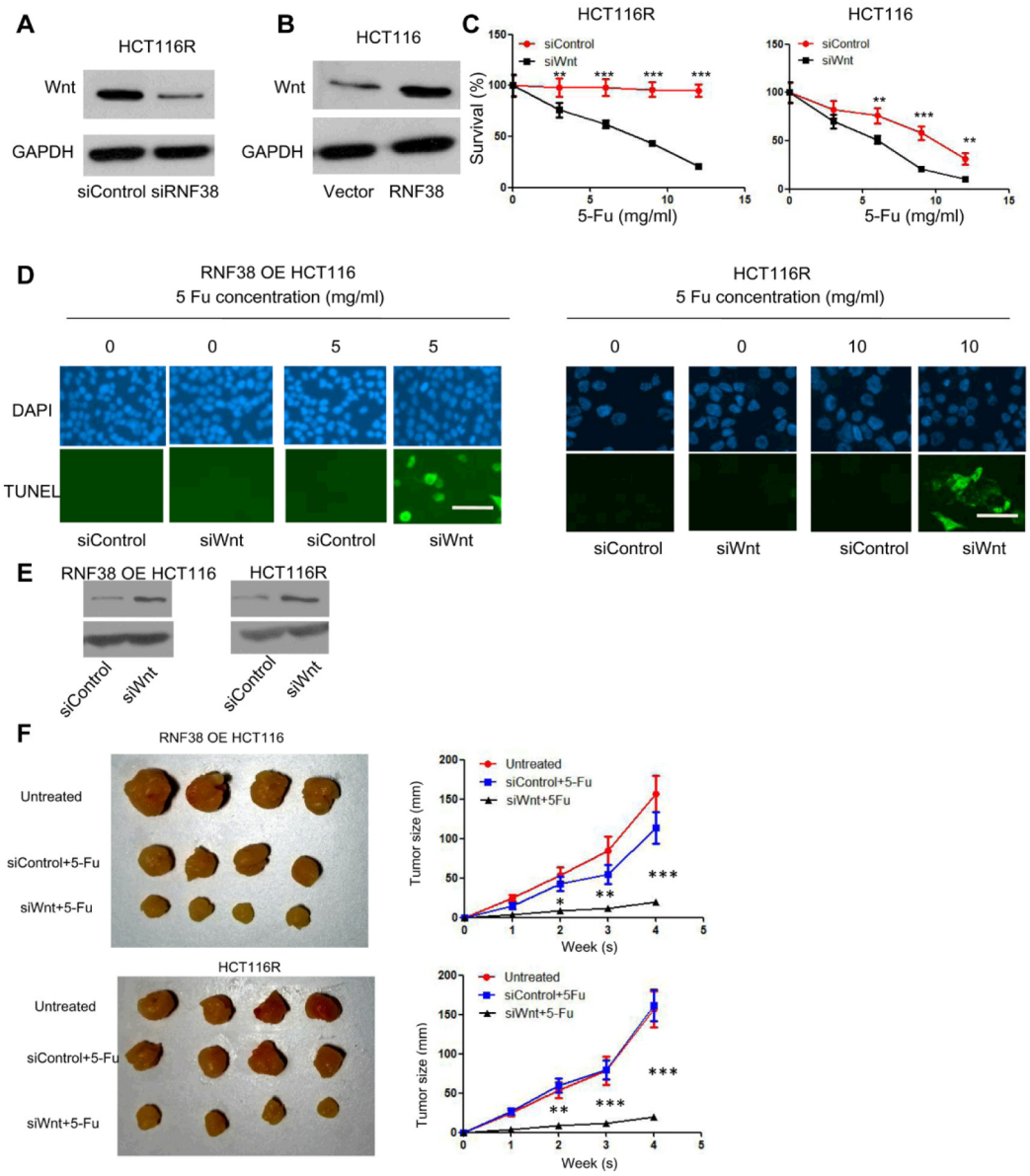

Figure 4. Wnt signaling was involved in RNF38-mediated 5-FU-resistance. **A:** s Protein level of Wnt in HCT116R cells either with RNF38 knockdown or not. **B:** Protein level of Wnt in HCT116 cells either with RNF38 overexpression or not. **C:** Survival rate of 5-FU-treated HCT116R and HCT116 cells either with RNF38 knockdown or not. **D:** TUNEL assay showed the influence of silenced RNF38 on apoptosis in 5-FU-treated HCT116 cells overexpressing RNF38 and HCT116R cells. Magnification 200×. Scale bar=100 μm. **E:** Protein level of p53 in HCT116 cells overexpressing RNF38 and HCT116R cells either with Wnt knockdown or not. **F:** Inactivation of the Wnt signaling reduced 5-FU resistance in CRC *in vivo*. Tumor size in nude mice administered with untreated HCT116 cells overexpressing RNF38, or HCT116 cells overexpressing RNF38 with either Wnt knockdown or not, followed by 5-FU administration (upper). Tumor size in nude mice administered with untreated HCT116R cells, or HCT116R cells with either Wnt knockdown or not, followed by 5-FU administration (bottom). *p<0.05; **p<0.01; ***p<0.001.

cell phenotypes [14-16]. A relevant clinical trial has shown the potential of Wnt as a target against 5-FU resistance [17]. In that paper, the Wnt signaling was identified to be activated in 5-FU resistant CRC cells. Notably, silence of Wnt markedly enhanced 5-FU sensitivity in nude mice bearing CRC. Nevertheless, how the Wnt signaling contributed to 5-FU resistance is still unclear.

As a famous tumor-suppressor gene, p53 deficiency in transgenic mice activates the Wnt signaling [18]. Inactivated p53 in 5-FU resistant cancer cells demonstrates the role of p53 in 5-FU resistance. Here, p53 was downregulated in HCT116R cells or those overexpressing RNF38 overexpressed cells. It is suggested that RNF38 overexpression suppressed p53 and activated Wnt signaling, thus leading to 5-FU resistance in CRC. We believe that RNF38 can be utilized as a therapeutic target in 5-FU-resistant CRC.

Conclusions

Overexpression of RNF38 enhanced 5-FU resistance in CRC. Furthermore, Wnt signaling was activated by RNF38 and involved in 5-FU resistance in CRC.

Conflict of interests

The authors declare no conflict of interests.

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