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ORIGINAL ARTICLE

RNF6 enhances radioresistance in colorectal cancer *via* activating the Wnt pathway

Xi Zhong[1], Bin Zhou[2], Zhaoying Lv[2], Yong Liu[3]

[1]Department of Critical Care Medicine, West China Hospital of Sichuan University, Chengdu, China. [2]Institute of Digestive Surgery and State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, Chengdu, China. [3]Department of Gastroenterologic Surgery, West China Hospital, Sichuan University, Chengdu, China.

Summary

Purpose: *RNF6 is verified to promote the malignant growth of colorectal cancer (CRC) and its level is linked to prognosis in CRC patients. Radioresistance is a key factor influencing prognosis in CRC. This study aimed to uncover the potential regulation of ring finger protein 6 (RNF6) in CRC radioresistance.*

Methods: *RNF6 levels in radioresistant and non-radioresistant CRC patients were detected. In vitro and in vivo regulatory effects of RNF6 on radioresistant CRC cell lines and nude mice bearing radioresistant CRC were examined, respectively. The involvement of Wnt pathway in CRC radioresistance was explored by Western blot.*

Results: *RNF6 was highly expressed in radioresistant CRC species than that of non-radioresistant ones. Identically, RNF6 was upregulated in radioresistant CRC cells compared to parental cells. SW1116 cells overexpressing RNF6 were more tolerant to radiotherapy, and similar results were obtained in nude mice bearing radioresistant CRC with overexpression of RNF6. Moreover, the Wnt pathway was activated during RNF6-induced radioresistance improvement in CRC.*

Conclusions: *RNF6 enhances radioresistance of CRC through activating the Wnt pathway.*

Key words: *colorectal cancer (CRC), radioresistance, RNF6, Wnt*

Introduction

Colorectal cancer (CRC) is a globally frequent cancer. There are 1.8 million newly cases of CRC and 800,000 die of this cancer each year [1]. CRC patients in the early stage can get a satisfactory prognosis after surgery. Nevertheless, advanced CRC is hard to be totally resected even after surgery and postoperative radiotherapy [2-4]. Of note, radioresistance is a vital cause of treatment failure in CRC [5].

Radiotherapy has been extensively applied in anti-tumor treatments [6]. It kills cancer cells through damaging double-stranded DNAs [7]. However, some cancer cells can survive and further metastasize to local and distant organs, resulting in a poor prognosis [8]. It is reported that malignant proliferative potential of radioresistant cancer cells is stronger than those of parental cells [9]. Therefore, it is of significance to clarify the molecular mechanisms of radioresistance in CRC.

Ring finger protein 6 (RNF6) locates on chromosome 13q12.13, where is often amplified in cancer tissues [10,11]. As a RING-type E3 ubiquitin-protein ligase, RNF6 is a member of the RNF family that regulates proteasomal degradation through ubiquitinating its target genes [12]. A relevant study demonstrated that overexpression of RNF6 deteriorates the development of CRC through the Wnt pathway [13]. The biological role of RNF6 in radioresistance of CRC, however, remains unclear.

Corresponding author: Yong Liu, MD. Department of Gastroenterologic Surgery, West China Hospital, Sichuan University, No. 37, Guoxue Road., Chengdu, Sichuan 610041, China.
Tel: +86 013408626064; Email: beckmanliuyong@163.com
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In this article, we first detected RNF6 levels in radioresistant and non-radioresistant CRC patients. Through establishing radioresistant CRC model in SW1116 cells and nude mice, we further illustrated the role of RNF6 and the Wnt pathway in radioresistance of CRC.

Methods

CRC species

Radioresistant CRC species (n=9) and non-radioresistant CRC species (n=9) were obtained from West China Hospital, Sichuan University. CRC patients of the sample collected did not receive anti-tumor treatment. This trial was approved by Ethics Committee of West China Hospital, Sichuan University.

Cell culture and establishment of radioresistant cell line

SW1116 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (10566024, Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (16000044, Gibco, Rockville, MD, USA), 100 U/mL penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA) in a 5% CO_2 incubator at 37°C.

SW1116 cells were irradiated at 2 Gy per day using an RS 2000 X-ray Biological Irradiator (Rad Source Technologies, St. Louis, MO, USA) for consecutive 30 days. The remaining alive cells were cultured for another 30 days, and then, radioresistant SW116 cells were obtained.

Cell proliferation assay

Colony formation assay: 500 treated cells were inoculated in each well of 6-well plates and cultured for 15 days. Visible colonies were treated in pre-cold ethanol for 1 h, dyed using Giemsa and counted.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay: 500 treated cells were inoculated in each well of 96-well plates. Twenty μL of MTT was applied in each well at the appointed time points and four hours later, 100 μL of dimethyl sulfoxide (DMSO) was added. The absorbance was measured at 490 nm using a spectrophotometric plate reader (Tecan, Waltham, MA, USA).

Establishment of stably expressing RNF6 cell line

For establishing SW1116 cell line stably expressing RNF6, SW1116 cells were transfected with lentivirus-packed pcmv-RNF6 plasmids (Abnova, Taipei, Taiwan), and controls were established by transfecting the control vector. After one-week cell culture, Puromycin was applied for selecting stably expressed cells.

Western blot

Cells were lysed for isolating cellular protein and electrophoresed. Protein samples were loaded on polyvinylidene fluoride (PVDF) membranes (Roche, Basel, Switzerland). Subsequently, non-specific antigens were blocked in 5% skim milk for 2 h. Membranes were reacted with primary [rabbit anti-RNF6 (PA5-58746, Invitrogen, Carlsbad, CA, USA), rabbit anti-Wnt (PA5-96453), rabbit anti-PCNA (MA5-11358) and rabbit anti-cyclin D3 (PA5-86078)] and secondary antibodies for indicated time. Band exposure and analyses were finally conducted.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Extracted RNAs by RNeasy Mini Kit (Qiagen, Hilden, Germany) were reversely transcribed using High Capacity complementary DNA (cDNA) Reverse Transcriptase kit (Applied Biosystems, Foster City, CA, USA). Genes expression levels were determined by quantitative PCR with SYBR Green Master Mix kit (Thermo Fisher, Waltham, MA, USA) using the $2^{-\Delta\Delta Cq}$ method. RNF-6, forward: TCGCATCGAAATTCGTAACGCTAT, reverse: GCCAATGCCTAGCGCATTGCCG; glyceraldheyde 3-phosphate dehydrogenase (GAPDH), forward: CAATGACCCCTTCATTGACC, reverse: GACAAGCTTCCCGTTCTCAG.

Animal procedures

Four-week-old nude mice were injected with 1×10^7 SW1116 cells stably expressing RNF6, SW1116 cells with RNF6 overexpression and Wnt knockdown, or controls on the left side of the back. One week later after cells injection, mice were irradiated with 4 Gy once a day for consecutive 3 days. Tumor size was recorded every week. Mice were euthanized at 28 days. This study was approved by the Animal Ethics Committee of Sichuan University.

Statistics

Data were expressed as mean ± standard deviation SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analyses. Differences between groups were compared using the Students t-test. Two-tailed $p<0.05$ was considered statistically significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Results

RNF6 was upregulated in radioresistant CRC species

Compared with non-radioresistant CRC species, RNF6 was highly expressed in radioresistant CRC species (Figure 1A). To further verify this finding, we established radioresistant SW1116 cell line. Compared with its parental cell line, RNF6 was upregulated in radioresistant CRC cells as well (Figure 1B). This suggested that RNF6 may be linked to radioresistance of CRC.

Overexpression of RNF6 enhanced radioresistance in CRC cells

To uncover the influence of RNF6 on radioresistance of CRC, the proliferative potential in radioresistant SW1116 cells was examined. MTT assay uncovered a higher viability in radioresistant SW1116 cells overexpressing RNF6 than those of controls. Meanwhile, colony number increased after overexpression of RNF6 in radioresistant cells

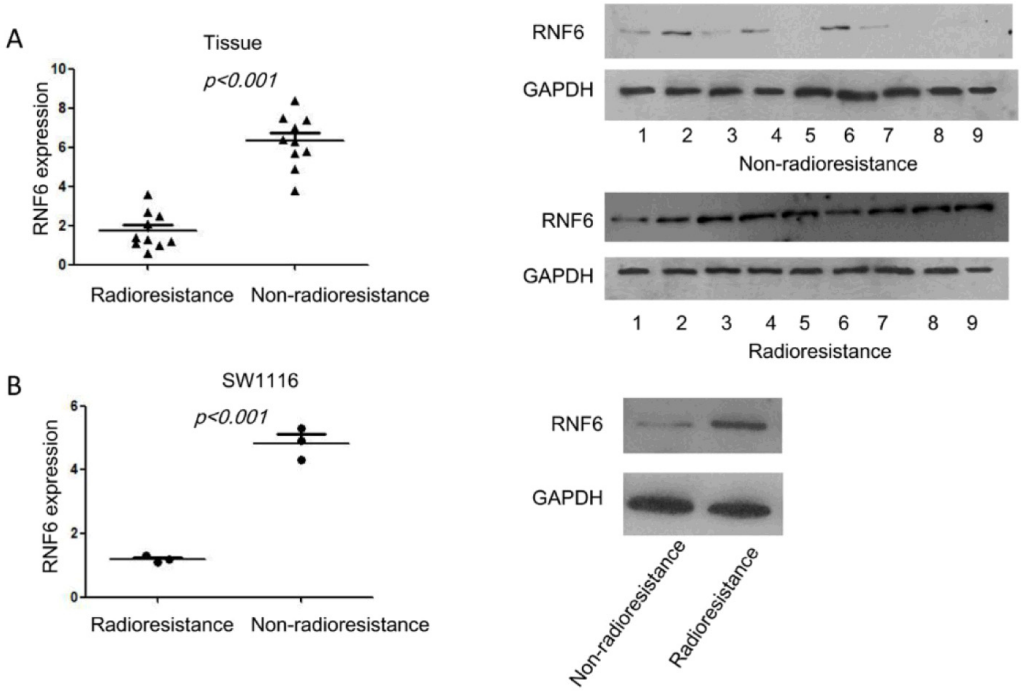

Figure 1. RNF6 was upregulated in radioresistant CRC species. **A:** RNF6 levels in radioresistant and non-radioresistant CRC species. **B:** RNF6 levels in radioresistant and non-radioresistant SW1116 cells.

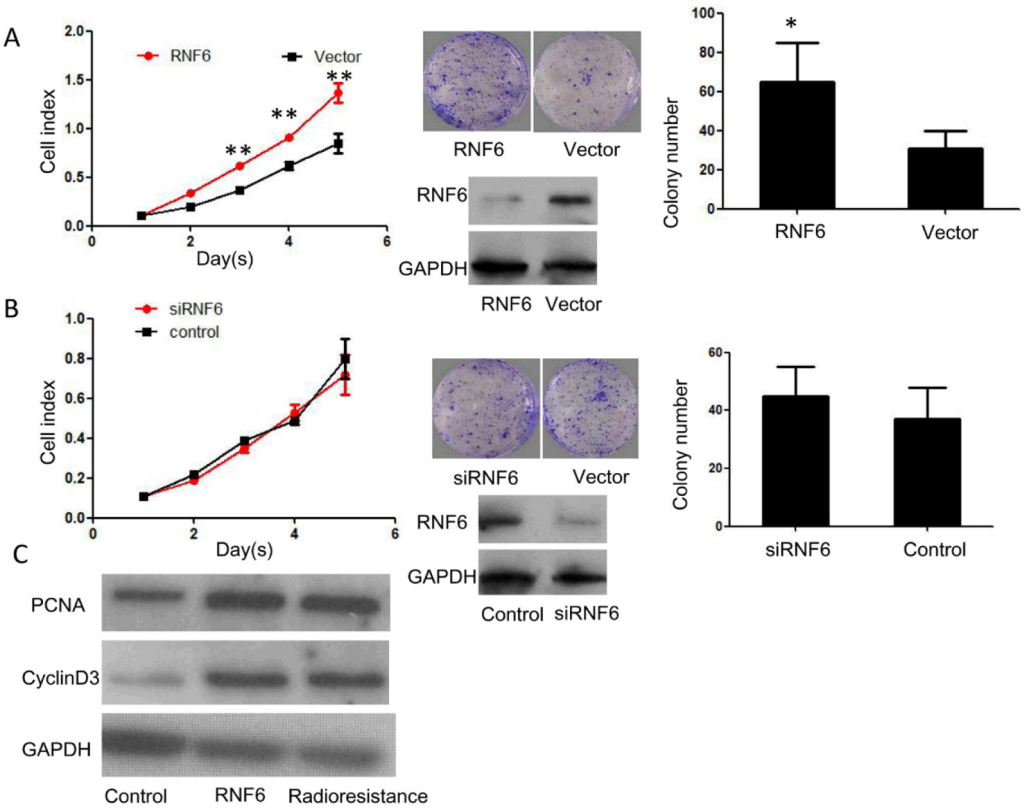

Figure 2. Overexpression of RNF6 enhanced radioresistance in CRC cells. **A:** MTT and colony formation showed viability and colony number in radioresistant SW1116 cells overexpressing RNF6 or not, respectively. (magnification: 10×). **B:** MTT and colony formation showed viability and colony number in radioresistant SW1116 cells with RNF6 knockdown or not, respectively. (magnification: 10×). C: Protein levels of PCNA and CyclinD3 in non-radioresistant SW1116 cells, radioresistant SW1116 cells overexpressing RNF6 or not (*p<0.05, **p<0.01).

(Figure 2A). Nevertheless, knockdown of RNF6 did not alter the proliferative potential in radioresistant CRC cells (Figure 2B). Furthermore, proliferation-associated genes were detected. As Western blot results showed, overexpression of RNF6 upregulated PCNA and CyclinD3 in radioresistant CRC cells (Figure 2C).

Overexpression of RNF6 enhanced radioresistance in nude mice bearing CRC

Next, we explored the role of RNF6 in enhancing radioresistance of CRC *in vivo*. Nude mice bearing CRC were irradiated for establishing CRC radioresistance model which showed that tumor size of CRC under irradiation was larger in mice overexpressing RNF *in vivo* than those of controls (Figure 3A). Moreover, higher protein levels of PCNA and CyclinD3 in radioresistant CRC mice overexpressing RNF6 further verified our findings (Figure 3B).

RNF6 activated the Wnt pathway

A previous study has reported that RNF6 is able to stimulate the malignant growth of CRC through activating the Wnt pathway. Here, the protein level of Wnt was markedly higher in radioresistant CRC species than controls (Figure 4A). Knockdown of

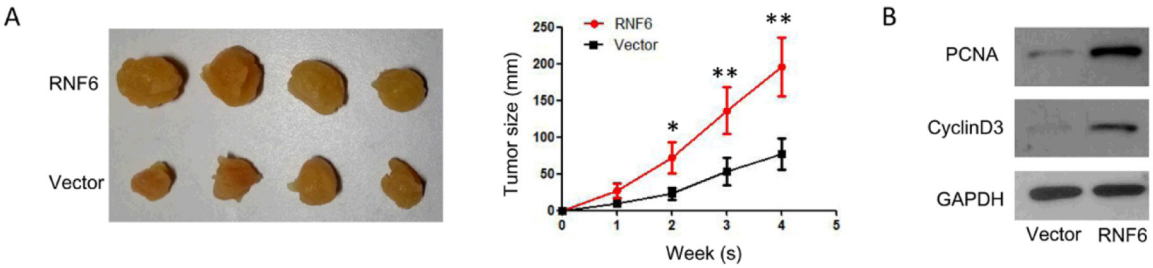

Figure 3. Overexpression of RNF6 enhanced radioresistance in nude mice bearing CRC. **A:** Tumor size in CRC resistant nude mice overexpressing RNF6 or not. **B:** Protein levels of PCNA and CyclinD3 in CRC resistant nude mice overexpressing RNF6 or not (*p<0.05, **p<0.01).

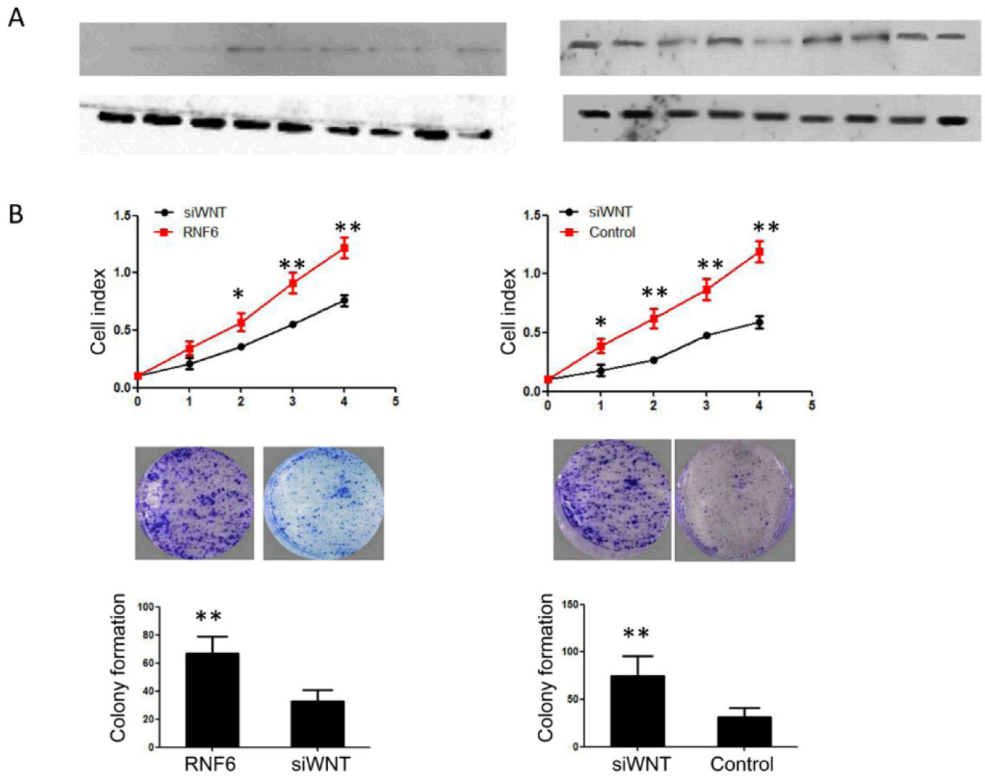

Figure 4. RNF6 activated the Wnt pathway. **A:** Protein level of Wnt in radioresistant and non-radioresistant CRC species. **B:** MTT and colony formation showed regulatory effect of silenced Wnt on viability and colony number in radioresistant SW1116 cells overexpressing RNF6, respectively. (magnification: 10×). MTT and colony formation showed regulatory effect of silenced Wnt on viability and colony number in radioresistant SW1116 cells, respectively (*p<0.05, **p<0.01).

Figure 5. Inhibition of the Wnt pathway suppressed the growth of CRC. Regulatory effect of silenced Wnt on tumor size in CRC resistant nude mice overexpressing RNF6 or not (**p<0.01).

Wnt decreased both viability and colony number in radioresistant SW1116 cells either overexpressing RNF6 or not (Figure 4B).

Inhibition of the Wnt pathway suppressed the growth of CRC

To explore the involvement of Wnt in CRC growth, a group of nude mice were injected with SW1116 cells with RNF6 overexpression and Wnt knockdown. These mice presented a similar growth rate of CRC compared with controls. However, tumor growth was markedly alleviated in these mice compared with those overexpressing RNF6 (Figure 5), suggesting that inhibition of the Wnt pathway protected malignant growth of radioresistant CRC.

Discussion

The increased incidence of CRC is closely linked to the altered modern lifestyle. Radiotherapy is preferred for CRC patients, especially in advanced stage patients since surgery is unable to totally resect cancer tissues [14]. However, radioresistance remarkably limits the therapeutic efficacy [15]. Radioresistance has become a major reason for metastasis and recurrence of CRC [8].

In this article, we have verified that RNF6 was a vital regulator in radioresistance of CRC. RNF6 is previously reported to be highly expressed in CRC species [13]. Notably, our findings showed highly expressed RNF6 in radioresistant CRC species than that of non-radioresistant ones, suggesting that RNF6 is closely linked to radioresistance of CRC. Subsequently, *in vitro* experiments demonstrated that overexpression of RNF6 accelerated the proliferative potential of radioresistant CRC cells. In addition, upregulated proliferation-associated genes also proved this finding, which was consistent with previous reports [9]. *In vivo* radioresistant CRC model also confirmed the role of RNF6 in stimulating the proliferative potential.

The Wnt pathway is a well-known cancer-related pathway [16]. RNF6 is identified to trigger the proliferative potential of CRC *via* activating the Wnt pathway [13]. Here, we found upregulated Wnt in radioresistant CRC species. Notably, knockdown of Wnt markedly decreased the proliferative rate in radioresistant CRC cells, showing that the Wnt pathway was involved in RNF6-regulated radioresistance of CRC.

Conclusions

RNF6 enhances radioresistance of CRC through activating the Wnt pathway. RNF6 may be a biomarker of CRC resistance.

Conflict of interests

The authors declare no conflict of interests.

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