RTKN2 Enhances Radioresistance in Gastric Cancer through Regulating the Wnt/β-Catenin Signalling Pathway

(Rhotekin 2 / gastric cancer / radiosensitivity / proliferation / migration / invasion / apoptosis / Wnt/β-catenin)

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Abstract. Adjuvant therapy and radiotherapy improves the survival of patients with metastatic and locally advanced gastric cancer (GC). However, the resistance to radiotherapy limits its clinical usage. Rhotekin 2 (RTKN2) functions as an oncogene and confers resistance to ultraviolet B-radiation and apoptosis-inducing agents. Here, the role of RTKN2 in radiosensitivity of GC cell lines was investigated. RTKN2 was found to be elevated in GC tissues and cells. A series of functional assays revealed that over-expression of RTKN2 induced GC cell proliferation, promoted GC cell migration and invasion, while inhibiting GC cell apoptosis. However, silence of RTKN2 promoted GC cell apoptosis, while repressing GC cell proliferation, invasion and migration. GC cells were exposed to irradiation, and data from cell survival and apoptotic assays showed that knock-down of RTKN2 enhanced radiosensitivity of GC through up-regulation of apoptosis and down-regulation of proliferation in irradiation-exposed GC cells. Moreover, the protein expression of β-catenin and c-Myc in GC cells was enhanced by RTKN2 over-expression, but reduced by RTKN2 silence. Interference of RTKN2 down-regulated nuclear β-catenin expression, while up-regulating cytoplasmic β-catenin in GC. In conclusion, RTKN2 contributed to cell growth and radioresistance in GC through activation of Wnt/β-catenin signalling.

Introduction

Gastric cancer (GC) is a malignant tumour with high incidence and poor prognosis (Sitarz et al., 2018). The prevalence of GC tends to increase in younger persons due to Helicobacter pylori infection, increased work pressure and changes in the diet structure (Holubiuk and Imiela, 2016). Surgical resection, radiotherapy and chemotherapy are widely used in the treatment of GC (Sitarz et al., 2018). However, patients are generally diagnosed at advanced stage due to the lack of early diagnostic biomarker, and particularly, patients with metastatic GC show resistance to the current therapies (Necula et al., 2019). Therefore, novel therapeutic targets for the clinical treatment of GC or amelioration of radiosensitivity might improve the poor prognosis of GC patients.

Rhotekin 2 (RTKN2) belongs to Rho GTPase family and functions as a scaffold protein that binds to Rho, thus regulating distinct cellular processes (Collier et al., 2004). RTKN2 has been shown to play an important role in regulating tumour progression. For example, RTKN2 was found to be a prognostic factor for non-small-cell lung cancer (Guo et al., 2021) and promoted tumorigenesis (Ji et al., 2020). RTKN2 was also involved in miR-181-mediated suppression of ovarian cancer (Lin et al., 2018). Silence of RTKN2 inhibited cell proliferation in bladder cancer (Liao et al., 2016) and promoted cell apoptosis in osteosarcoma (Wang et al., 2018). However, the functional role of RTKN2 has not been reported in GC, and its related mechanism is unclear. Moreover, RTKN2 promoted survival of human embryonic kidney cells exposed to intrinsic apoptotic agents, camptothecin and 25-hydroxy cholesterol (Collier et al., 2009). RTKN2 also protected keratinocytes against ultraviolet B-radiation-induced cell apoptosis (Li et al., 2014). Therefore, RTKN2 might also be involved in the radioresistance of GC.

Wnt signalling is a key pathway to control cell development and is correlated with cancer progression (Zhang and Wang, 2020). The Wnt pathway is activated in several cancer types (Perez-Plasencia et al., 2020), and in-
hibition of Wnt is considered to be a promising strategy for the prevention of GC (Chiurillo, 2015). In addition, the Wnt pathway is also implicated in radioresistance of tumour cells (Wu et al., 2016). Suppression of Wnt/β-catenin signalling contributed to the radiosensitivity of gastric adenocarcinoma (Sun et al., 2021). RTKN2 promoted Wnt/β-catenin signalling and promoted cell proliferation in colon cancer (Pang et al., 2017). Therefore, RTKN2 was hypothesized to regulate the progression and radiosensitivity of GC cells through Wnt/β-catenin signalling. In this study, the effects of RTKN2 on GC cell proliferation, invasion and migration, as well as radiosensitivity, were investigated.

Material and Methods

Bioinformatic analysis

GEPIA (http://gepia.cancer-pku.cn/) was used to detect the expression of RTKN2 in GC tissues (N = 408) and normal tissues (N = 211). GSE118897 gene expression profiling data was downloaded from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/). The GSE118897 dataset contains 10 GC samples and 10 normal gastric mucosa tissue samples, and the expression of RTKN2 was then analysed.

Cell culture

GC cell lines (AGS, SNU-1, NCI-N87 and HGC-27) and a gastric epithelial cell line (GES-1) were purchased from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) containing 10 % foetal bovine serum (Invitrogen, Carlsbad, CA, USA). Cells were incubated in a 37 °C incubator with 5 % CO2.

qRT-PCR

GC cell lines and GES-1 were lysed in a TRIzol kit (Invitrogen, Carlsbad, CA, USA), and the isolated RNAs were then translated into cDNAs. A PreQ1 II kit (Takara, Dalian, Liaoning, China) was used to determine RTKN2 mRNA expression with the following primers (forward: 5’-ACAGTTCGCGTTGGAGATGG AG-3’ and reverse: 5’-GTCGAGCATTGCACACCATG AG-3’). The relative expression of RTKN2 was calculated using the 2ΔΔCq method through normalization to GAPDH (forward: 5’-TCAACGACCACTTTGTCAAAG CAGAGT-3’and reverse: 5’-GCTGGTGGTCCAGGGG TCTTACT-3’).

Cell transfection and treatment

Full-length RTKN2 DNA was subcloned into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA) and named RTKN2. shRNA targeting RTKN2 (shRTKN2) and the negative control (shNC) were also synthesized by Invitrogen. The pcDNA vectors (RTKN2 and the negative control, pcDN empty vector) and shRNAs were transfected into AGS cells using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) for 24 hours. Cells were then exposed to 2, 4, 6, or 8 Gy irradiation under X-ray irradiator RS2000 (RAD SOURCE, Suwanee, GA, USA) at a linear rate of 1.15 Gy/min.

Cell viability and proliferation assays

AGS cells were seeded into 96-well plates and transfected with pcDNA vectors and shRNAs for 24 hours. Cells were exposed to irradiation (2, 4, 6, or 8 Gy) at a linear rate of 1.15 Gy/min and then grown for 24 hours. Cells were incubated with 10 μl CCK8 solution (Beyotime, Beijing, China) for 2 hours. Absorbance at 450 nm was measured by a microplate reader (Thermo Fisher Scientific). For colony-formation assay, AGS cells were seeded into 6-well plates and transfected with pcDNA vectors and shRNAs for 24 hours. Cells were exposed to 4 Gy irradiation at a linear rate of 1.15 Gy/min, cultured in the RPMI-1640 medium for 10 days, and then fixed in methanol. Following staining with crystal violet, cells were observed under a microscope (Olympus, Tokyo, Japan), and colony numbers were calculated by Image J v.1.46 (National Institutes of Health, Bethesda, MD, USA).

Cell apoptosis assay

AGS cells were transfected with pcDNA vectors and shRNAs for 24 hours and then exposed to 4 Gy irradiation at a linear rate of 1.15 Gy/min. Cells were grown for 24 hours, harvested, and then resuspended in binding buffer from Dead Cell Apoptosis Kits with Annexin V for Flow Cytometry (Thermo Fisher Scientific, Waltham, MA, USA). Cells were stained with 5 μl of propidium iodide (PI) and 5 μl of FITC-labelled annexin V. A FACS flow cytometer (Life Technologies, Darmstadt, Germany) was used to evaluate the apoptotic rate according to PI and annexin V-positive cells.

Cell migration and invasion assays

AGS cells were transfected with pcDNA vectors and shRNAs for 24 hours and then exposed to 4 Gy irradiation at a linear rate of 1.15 Gy/min. Cells were grown for 24 hours, suspended in 100 μl serum-free RPMI-1640 medium, and then plated into the upper Matrigel-coated Transwell insert chamber (Corning Incorporated, Corning, NY, USA). Medium with 15 % foetal bovine serum (400 μl) was added into the lower chamber. Twenty-four hours later, cells in the upper chamber were removed, and cells in the lower chamber were stained with crystal violet and then observed under a microscope to detect the invasive cells (Olympus). For detection of cell migration, AGS cells post transfection and 4 Gy irradiation in serum-free RPMI-1640 medium were also plated into the upper Transwell insert chamber without Matrigel coat, and underwent the same experiment as mentioned previously. The numbers of migratory cells were also observed under the microscope.
Western blot

GES-1 and GC cell lines were lysed in RIPA buffer (Beyotime, Beijing, China), and the nuclear and cytoplasmic proteins were isolated using NE-PER cytoplasmic and nuclear extraction reagents (Thermo Fisher Scientific, Waltham, MA, USA). Protein concentration was determined by a BCA kit (Thermo Fisher Scientific, Waltham, MA, USA), and the protein samples were then separated by 10% SDS-PAGE. Following transfer onto nitrocellulose membranes and blocking in 5% bovine serum albumin, the membranes were probed with specific antibodies: anti-RTKN2 and anti-β-actin (1 : 2000), anti-MMP2 and anti-MMP9 (1 : 2500), anti-β-catenin and anti-c-Myc (1 : 3000), anti-β-tubulin and anti-histone H3 (1 : 3500). The membranes were then washed and incubated with horseradish peroxidase-conjugated secondary antibody (1 : 4000). All the antibodies were from Abcam, Cambridge, MA, USA. Immunoreactivities were visualized using enhanced chemiluminescence (Sigma-Aldrich, St. Louis, MO, USA).

Statistical analysis

All data with at least triple replicates were expressed as mean ± SEM and analysed by Student’s t-test or one-way analysis of variance under SPSS software. A P value of < 0.05 was considered as statistically significant.

Results

Up-regulation of RTKN2 in GC cells

Data based on TCGA (Fig. 1A) and GSE118897 (Fig. 1B) showed that RTKN2 was elevated in GC tissues compared to that in the normal tissues. Similarly, GC cell lines (AGS, SNU-1, NCI-N87, HGC-27) also expressed higher RTKN2 mRNA (Fig. 1C) and protein (Fig. 1D) than the gastric epithelial cell line (GES-1), demonstrating its potential role in GC.

RTKN2 enhanced radioresistance of GC cells

The AGS cell line was then transfected with pcDNA vectors or shRNAs to up-regulate or down-regulate the protein expression of RTKN2, respectively (Fig. 2A). Transfection with pcDNA-RTKN2 increased the viability of AGS exposed to irradiation, while transfection with shRTKN2 reduced the viability of irradiation-exposed AGS cells (Fig. 2B). Colony numbers of AGS cell were promoted by RTKN2 over-expression while suppressed by knock-down of RTKN2 (Fig. 2C and D).

Fig. 1. Up-regulation of RTKN2 in GC cells

(A) RTKN2 was up-regulated in GC tissues (red) compared to the normal tissues (black) based on the TCGA database. (B) RTKN2 was up-regulated in GC tissues (orange) compared to the normal tissues (green) based on GSE118897. (C) RTKN2 mRNA was up-regulated in GC cell lines (AGS, SNU-1, NCI-N87 and HGC-27) (orange) compared to the gastric epithelial cell line (GES-1) (green). (D) RTKN2 protein was up-regulated in GC cell lines (AGS, SNU-1, NCI-N87 and HGC-27) (orange) compared to the gastric epithelial cell line (GES-1) (green). * P < 0.05, ** P < 0.01 and *** P < 0.001.
Besides, over-expression of RTKN2 enhanced the colony formation of irradiation-exposed AGS cells, while it was reduced by silence of RTKN2 (Fig. 2C and D). Moreover, over-expression of RTKN2 repressed AGS cell apoptosis, and knock-down of RTKN2 promoted AGS cell apoptosis (Fig. 2E). The apoptosis of irradiation-exposed AGS cells was also repressed by over-expression of RTKN2 whereas promoted by knock-down of RTKN2 (Fig. 2E). These results suggested that the silence of RTKN2 enhanced the radiosensitivity of GC cells through inhibition of proliferation and promotion of apoptosis.

RTKN2 enhanced invasion and migration of GC cells

The invasion and migration of AGS cells was promoted by RTKN2 over-expression while suppressed by silence of RTKN2 (Fig. 3A). Furthermore, over-expression of RTKN2 increased the protein expression of MMP2 and MMP9 in AGS cells, while knock-down of RTKN2 decreased expression of these proteins (Fig. 3B). These results indicated that the silence of RTKN2 reduced the invasion and migration of GC cells.

Fig. 2. RTKN2 enhanced radioresistance of GC cells.
(A) AGS cells were transfected with pcDNA vectors (orange) or shRNAs (purple) to up-regulate and down-regulate the protein expression of RTKN2. (B) Transfection with pcDNA-RTKN2 (orange) increased the viability of irradiation-exposed AGS cells, while it was reduced by transfection with shRTKN2 (purple). (C) Transfection with pcDNA-RTKN2 (orange) increased proliferation of irradiation-exposed AGS cells, while it was reduced by transfection with shRTKN2 (purple). (D) Transfection with pcDNA-RTKN2 (orange) increased the colony numbers of irradiation-exposed AGS cells, while they were reduced by transfection with shRTKN2 (purple). (E) Transfection with pcDNA-RTKN2 (orange) suppressed the apoptosis of irradiation-exposed AGS cells, while it was promoted by transfection with shRTKN2 (purple). * P < 0.05, ** P < 0.01, *** P < 0.001.
**RTKN2 enhanced activation of Wnt/β-catenin signalling in GC cells**

The protein expression of β-catenin and c-Myc in AGS cells was up-regulated by over-expression of RTKN2 while down-regulated by knock-down of RTKN2 (Fig. 4A). Additionally, over-expression of RTKN2 reduced the cytoplasmic β-catenin expression while enhanced the nuclear β-catenin expression (Fig. 4B). However, the silence of RTKN2 inhibited the activation of Wnt/β-catenin signalling through up-regulating the cytoplasmic β-catenin and down-regulating the nuclear β-catenin in AGS cells (Fig. 4B).

**Discussion**

A previous study has shown that RTKN promoted up-regulation of histone deacetylase 1 to reduce the acetylation level of p53, thus suppressing GC cell cycle progression and apoptosis (Sun et al., 2019). Moreover, RTKN enhanced the resistance of GC cells to interferon-α-induced tumour suppression (Yin et al., 2012). This study investigated the effects of RTKN2 on cell proliferation, invasion, migration and radioresistance of GC.

Consistently, this study demonstrated up-regulation of RTKN2 in GC tissues and cells. Knock-down of RTKN2 suppressed GC cell proliferation and metastasis, and promoted GC cell apoptosis, suggesting the oncogenic role of RTKN2 in GC. Moreover, a previous study has shown that RTKN2 contributed to cell migration and invasion in non-small-cell lung cancer through up-regulating MMP2 and MMP9 (Ji et al., 2020). Here, the protein expression of MMP2 and MMP9 in GC cells was down-regulated by silence of RTKN2, suggesting that RTKN2 might contribute to the metastasis in GC.

RTKN has been shown to enhance chemoresistance of GC cells to paclitaxel and 5-fluorouracil through activation of NF-κB signalling (Liu et al., 2004). Con-

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**Fig. 3. RTKN2 enhanced metastasis of GC cells.**

(A) The invasion and migration of AGS cells was promoted by RTKN2 over-expression (orange) while suppressed by RTKN2 silence (purple). (B) Over-expression of RTKN2 (orange) increased the protein expression of MMP2 and MMP9 in AGS cells, while it was decreased by knock-down of RTKN2 (purple). **P < 0.01, ***P < 0.001.
sistenty, results in this study showed that knock-down of *RTKN2* aggravated irradiation-induced reduction of cell proliferation and enhancement of cell apoptosis in GC, confirming that *RTKN2* contributed to the radioresistance of GC.

Wnt/β-catenin signalling, essential for cell proliferation and differentiation, is dysregulated in GC and associated with tumour invasion (Ashrafizadeh et al., 2020). Wnt/β-catenin signalling also enhanced high-mobility group box 1 protein expression to promote DNA damage repair, thus promoting radioresistance in oesophageal squamous cell carcinoma (Zhao et al., 2018). Activation of Wnt/β-catenin signalling contributed to the radioresistance of GC (Chi et al., 2020). *RTKN2* was involved in Wnt/β-catenin pathway-mediated hepatocellular carcinoma progression (Huang et al., 2020), and silence of *RTKN2* reduced protein expression of β-catenin and c-Myc to suppress activation of Wnt/β-catenin signalling, which thus inhibited progression of colon cancer (Pang et al., 2017). Here, knock-down of *RTKN2* also down-regulated the protein expression of β-catenin and c-Myc in GC cells, enhanced cytoplasmic β-catenin expression and reduced nuclear β-catenin expression to inhibit the activation of Wnt/β-catenin signalling.

In conclusion, *RTKN2* functioned as an oncogene in GC. Silence of *RTKN2* reduced cell proliferation and metastasis in GC and sensitized GC cells to irradiation through inactivation of Wnt/β-catenin signalling. However, the effects of *RTKN2* on *in vivo* tumour growth and radiosensitivity of GC cells should be investigated in further research.

**Competing interests**

The authors state that there are no conflicts of interest to disclose.

**Contribution of authors**

Huigeng Zhao and Jiajun Yin designed the study and carried it out, Xi Chen and Ju Wu supervised the data collection, analysed the data, interpreted the data, Wei Wang and Liwang Tang prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

**References**


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**Fig. 4.** *RTKN2* enhanced activation of Wnt/β-catenin signalling in GC cells. (A) Protein expression of β-catenin and c-Myc in AGS cells was up-regulated by over-expression of *RTKN2* (orange) and down-regulated by knock-down of *RTKN2* (purple). (B) Over-expression of *RTKN2* (orange) reduced cytoplasmic β-catenin expression while enhancing nuclear β-catenin expression, while silence of *RTKN2* (purple) inhibited activation of Wnt/β-catenin signalling through up-regulating cytoplasmic β-catenin and down-regulating nuclear β-catenin in AGS cells. **P < 0.01, ***P < 0.001.


