ORIGINAL RESEARCH

Expression and biological function of rhotekin in gastric cancer through regulating p53 pathway

Meng-Yao Sun[1,*]
Hong Zhang[2,*]
Jie Tao[1]
Zhen-Hua Ni[1]
Qiu-Xue Wu[1]
Qing-Feng Tang[1]

[1]Department of Clinical Laboratory and Central Laboratory, Putuo Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai 200062, China; [2]Center for Innovative Chinese Medicine Research, Institute of Interdisciplinary Medical Sciences, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China

*These authors contributed equally to this work

Background/aim: Gastric cancer (GC) is one of a most threatening cancer globally. Rhotekin (RTKN), a Rho effector, has been reported to be upregulated in GC tissues. This study aimed to investigate the underlying regulatory roles of RTKN in the biological behavior of GC.

Methods: Real-time PCR and Western blotting were carried out to detect the mRNA and protein expression, respectively. Cell Counting Kit-8 and xenograft nude mice model were used to evaluate cell proliferation. Flow cytometry analysis was performed to assess cell cycle distribution and cell apoptosis.

Results: RTKN had high expression level in GC compared with normal tissues. RTKN expression strongly associated with tumor size, TNM stage, lymphnode metastasis and the poor prognosis of patients with GC. Downregulation of RTKN significantly repressed GC cell proliferation, but increased cell population in G1/S phase and induced cell apoptosis. Moreover, the RTKN expression level was related to the p53 signaling pathway and histone deacetylase (HDAC) Class I pathway. RTKN knockdown caused a notable increment in the acetylation level of p53 (Lys382), and the expression of p53-target genes (p21, Bax, and PUMA), as well as a reduction in the expression of a potential deacetylase for p53, HDAC1. Notably, downregulation of HDAC1 had similar effects as RTKN knockdown, and RTKN overexpression could hardly abrogate the effects of HDAC1 knockdown on GC cells.

Conclusion: RTKN could work as an oncogene via regulating HDAC1/p53 and may become a promising treatment strategy for GC.

Keywords: RTKN, gastric cancer, proliferation, invasion, HDAC1/p53

Introduction

Gastric cancer (GC) ranks among the top four cancer diagnoses and is the second most common cause of cancer-related death in the world.[1–3] In spite of advances made in the early detection and treatment of GC,[4,5] it is frequently not diagnosed until an advanced stage, and has a low survival rate.[6–8] The main risk factors for gastric cancer include *Helicobacter pylori* (*H pylori*) infection and dietary factors.[9,10] Beside environmental factors, GC is a complicated multistep process involving various oncogenic signaling pathways.[11,12] Until now, due to its high incidence rate, poor prognosis, and limited treatment options, GC remains a major clinical challenge to clinicians.

Rhotekin (RTKN) has been identified as a Rho effector protein. Rho is one of the best-characterized members of Rho GTPases, which play a critical role in multiple biological processes, including cytoskeleton reorganization, cell differentiation, cell cycle progression, and cell migration.[13] RTKN was initially found complexing with active form of Rho,[14] and subsequent studies identified several interaction partners of RTKN,

Correspondence: Qing-Feng Tang
Department of Clinical Laboratory and Central Laboratory, Putuo Hospital, Shanghai University of Traditional Chinese Medicine, 164 Lanxi Road, Shanghai 200062, China
Email tangqingfeng126@126.com

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including septin9b,[15] LIN7B,[16] PIST[17] and tax-interacting protein 1,[18,19] vinexin[20] and S100A4,[21] and suggested possible functions of RTKN in the formation and/or maintenance of septin filament, focal adhesions, stress fiber, and cell polarity. Evidence has suggested that RTKN is overexpressed in human cancer tissues, including GC[22] and bladder cancer,[23] when compared with corresponding normal tissues. RTKN overexpression in GC cells inhibited apoptosis, which was dependent on Rho activity and NF-κB activation.[22,24] p53, an important tumor suppressor located at 17pl3.1, has multiple biological functions in regulating cell cycle, inhibiting cell apoptosis, and maintaining genome stability via regulating the transcription of >150 target genes.[25,26] It has been known that loss of function of the p53 gene plays a central role in the development of cancers. Mutations in the p53 gene are the most common genetic alterations and have been reported in various human cancers including GC.[27–29] The acetylation levels of p53 correlate with the activation and stabilization of p53.[30]

In the current study, we confirmed the upregulation of RTKN in GC tissues, explored the association of RTKN expression with the aggressive characteristics and survival properties of GC patients, and investigated the functions of RTKN in GC cell proliferation, cell cycle arrest, and apoptosis. Besides, we explored that the p53 signaling pathway may be involved in the biological functions of RTKN in GC cells. Our data suggest that RTKN may be an effective oncogene and a therapeutic target for GC.

Materials and methods
GC tissue microarray

A tissue microarray (Cat#: HStm-A180Su-09, Shanghai Outdo Biotech, Shanghai, China) with 90 matched pairs of primary GC samples and adjacent gastric tissues was applied to evaluate the expression and clinical relevance of RTKN. Among these samples, one paired tumor and adjacent normal tissues were excluded due to incomplete information of the tissues. The core diameter on this tissue microarray was 1.5 mm.

Immunohistochemical staining

The sections were deparaffinized in xylene and rehydrated in ethanol, and then heated in 0.01 M citrate buffer (pH 6.0) by autoclave for 20 minutes. Subsequently, to inactivate endogenous peroxidases, the sections were incubated with 0.3% hydrogen peroxide for 30 minutes. After incubation with 10% normal goat serum to block nonspecific binding sites, the sections were probed with anti-RTKN (Abcam, Cambridge, MA, USA) overnight at 4°C, and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hour at room temperature. Finally, the sections were stained with the 3,3′-diaminobenzidine solution (Vector Laboratories, Burlingame, CA, USA) and counterstained with hematoxylin. The specimens were divided into RTKN high-expression group (≥25% of tumor cells were positively stained) and low-expression group (<25% of tumor cells were positively stained).

Cell culture

Human GC cell lines MKN-45, SGC-7901, MGC-803, and AGS were purchased from Cell Bank of Chinese Academy of Science (Shanghai, China). Cells were cultured and maintained in RPMI 1640 containing 10% (v/v) fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere of 5% CO_2 in air at 37°C.

Reverse transcription and real-time PCR

Total RNA was extracted from specimens or culture cells with Trizol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed to complementary DNA with oligo (dT) primers. Real-time PCR was then performed with the gene-specific primers and SYBR Green Master Mixes (Thermo Fisher Scientific, Waltham, MA, USA) on real-time PCR system (Thermo Fisher Scientific). The relative expression mRNA levels were normalized to GADPH expression. All primers were designed with Primer Premier 6 Software and are listed in Table 1.

Table 1 Primer pairs used for real-time PCR

Gene	Primers (forward/reverse)
RTKN	5′-GCCGCTGCTTACTATTGC-3′ and 5′-GTGCTTCCCGACTTTCTG-3′
HDAC1	5′-GCTCCACATCAGTCCTTCC-3′ and 5′-GGTCGTCTTCGTCCTCATC-3′
HDAC2	5′-AGGCAAATACTATGCTGTC-3′ and 5′-TGAAACAACCCAGTCTATC-3′
HDAC3	5′-CGGGATGGCATTGATGAC-3′ and 5′-GGGCAACATTTCGGACAG-3′
HDAC8	5′-CTGGTCCCGGTTTATATC-3′ and 5′-CGTCTTCTACACCATCTC-3′
p53	5′-GTGAGGGATGTTTGGGAGATG-3′ and 5′-CCTGGTTAGTACGGTGAAGTG-3′
P21	5′-TAGCAGCGGAACAAGGAG-3′ and 5′-AAACGGGAACCAGGACAC-3′
Bax	5′-CTGAGCGAGTGTCTCAAG-3′ and 5′-CAGCCCATGATGGTTCTG-3′
PUMA	5′-ATGGGACTCCTGCCCTTAC-3′ and 5′-TCCCTCTCCTGGCTTCTTG-3′
GAPDH	5′-AATCCCATCACCATCTTC-3′ and 5′-AGGCTGTTGTCATACTTC-3′

Western blot analysis

Cell treated as indicated were harvested. The protein concentration was analyzed by BCA protein Assay Reagent (Sangon Biotech, Shanghai, China). Equivalent amounts of whole protein (35 µg) were separated with 10% or 12% SDS-PAGE and then transferred onto nitrocellulose membranes. The membranes were blocked with 5% skimmed milk in Tris-buffered saline supplementing with 0.05% Tween-20 (TBST) at room temperature for 1 hour. Then, the membranes were incubated with primary antibodies according to the manufacturers' instructions. Following washing with TBST, the membranes were incubated with the HRP-conjugated secondary antibodies at room temperature for 1 hour. The membrane signals were detected using an enhanced chemiluminescence kit (BioRad, Richmond, CA, USA). Antibodies against RTKN, histone deacetylase (HDAC)1, acetylated p53 (Ace-p53), and p21 were from Abcam. Antibodies against p53 and GAPDH were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against Bax and Puma were from Santa Cruz (Santa Cruz, CA, USA).

Manipulation of RTKN expression by lentivirus

shRNA oligos targeting RTKN (shRTKN#1, CCGGGA-ACTGCGGTTAGAGCTGTATCTCGAGATAC-AGCTCTAACCGCAGTTCTTTTTC; shRTKN #2, CCGGGAAGCAGTGCTGTGATGAAATCTCGAGA-TTTCATCACAGCACTGCTTCTTTTTC; and shRTKN#3, CCGGAAGAACCCTTGGAGCAAACATCTCGAGAT-GTTTGCTCCAAGGGTTCTTTTTTTC) were annealed and cloned into AgeI- and EcoRI-digested pLKO.1 (Addgene, Cambridge, MA, USA). The full-length human RTKN was cloned into pLVX-puro (Clontech, Palo Alto, CA, USA). The lentivirus of shRTKN, control shRNA (shNC), pLVX-RTKN (OE), or pLVX-puro (Vector) was produced in 293 T cells along with packaging plasmids, psPAX2 and pMD2.G.

siRNA

HDAC1 siRNA duplex (siHDAC1, 5′-GGAAGAGUUCUC-CGAUUCU-3′) and control scrambled siRNA duplex (siNC, 5′-CCAUUCCGAUCCUGAUCCG-3′) were synthesized by Genepharma (Shanghai, China). siRNAs were transfected into cells following the manufacturer's instructions of Lipofectamine 2000 (Invitrogen).

Cell proliferation assay

After transduced with indicated lentivirus, the proliferation of MKN-45 and SGC-7901 cells was assessed by Cell Counting Kit (CCK)-8 kit (Beyotime, Shanghai, China). Briefly, cells were seeded in 96-well plates (4×10^3 per well), and further incubated for 12, 24, 48, or 72 hours. At 1 hour before the end point of incubation, cells were incubated with CCK-8 reagent. The OD values at a wavelength of 450 nm were determined by a microplate reader. Experiments were performed in triplicate and repeated at least three times.

Cell cycle and apoptosis assays

MKN-45 and SGC-7901 cells were treated as indicated. At 48 hours after treatment, the cells were harvested, washed with PBS, and proceeded to cell cycle and apoptosis assays. For cell cycle analysis, the cells were fixed in ethanol at −20°C overnight, washed with PBS, and stained with propidium iodide (PI, Sigma, St. Louis, MO, USA) at 37°C for 30 minutes. The cell cycle distribution was analyzed by using flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA). For cell apoptosis analysis, cells were stained with Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Beyotime), and apoptotic rates were also analyzed by using flow cytometry (BD Biosciences).

Growth of cells in athymic nude mice and tumor size determination

SGC-7901 cells stably transduced with shNC or shRTKN were established by puromycin selection. Twelve male athymic nude mice (4–6 weeks old) were randomly divided into two groups (six mice/group), and shNC and shRTKN cells were subcutaneously injected to the two groups of mice, respectively (2×10^6 cells per mouse). The tumor size was measured every 3 days after tumor formation (around 7–14 days). And 46 days later, the mice were sacrificed, and the tumors were collected and weighted on a digital balance.

Bioinformatics analysis

The microarray data were downloaded from The Cancer Genome Atlas website (TCGA, https://cancergenome.nih.gov/) for the GC project (Access id: TCGA-STAD-exp-HiSeq). RTKN expression was compared between GC tissues (n=249) and normal gastric mucosa (n=33). Gene set enrichment analysis (GSEA) was conducted as previously described based on TCGA dataset with the GSEA Software version 2.0 from the Broad Institute at MIT.[31]

Statistical analysis

Experimental data are presented as mean ± SD of three independent replicates. Statistical analysis was performed with GraphPad Prism Software 6.0 (SanDiego, CA, USA). The association between RTKN expression and clinicopatho-

logical features were assessed using the Fisher's exact test. Kaplan–Meier survival and log-rank analysis were done to determine the association between RTKN expression and overall survival of GC patients. Two-tailed Student's *t*-test was used to compare results between two groups, while one-way ANOVA followed by Dunnett's multiple comparison was used to compare among three or more groups. $P<0.05$ was considered significantly different.

Ethics approval and consent to participate

The use of human tissue microarray was approved by Ethics Committee of Putuo Hospital (Shanghai, China) in accordance with the Declaration of Helsinki. The animal experiments were approved by the Animal Research Committee in Putuo Hospital, Shanghai University of Traditional Chinese Medicine, and performed following the guidelines for the Care and Use of Experimental Animals of Shanghai University of Traditional Chinese Medicine.

Results

Upregulated RTKN expression correlated with poor prognosis of GC patients

We first analyzed RTKN expression using TGGA GC dataset, microarray data from ArrayExpress (Access id: TCGA-STAD-exp-HiSeq). As a result, RTKN mRNA expression was significantly higher in GC tissues than in normal gastric mucosa ($P<0.0001$, Figure 1A). Immunohistochemistry analysis in a GC tissue microarray that contains 89 pairs of GC tissues and adjacent normal tissues also indicated the upregulated RTKN expression in GC tissues. Further, the 89 GC patients were divided into two groups: RTKN high group (n=55) and RTKN low group (n=34; Figure 1B), and the association between RTKN expression levels and clinical characteristics was assessed. Notably, RTKN overexpression was strongly associated with tumor size ($P=0.0265$), TNM stage ($P=0.0162$), and lymph-node metastasis ($P=0.0010$; Table 2). Kaplan–Meier analysis showed that upregulated RTKN expression was
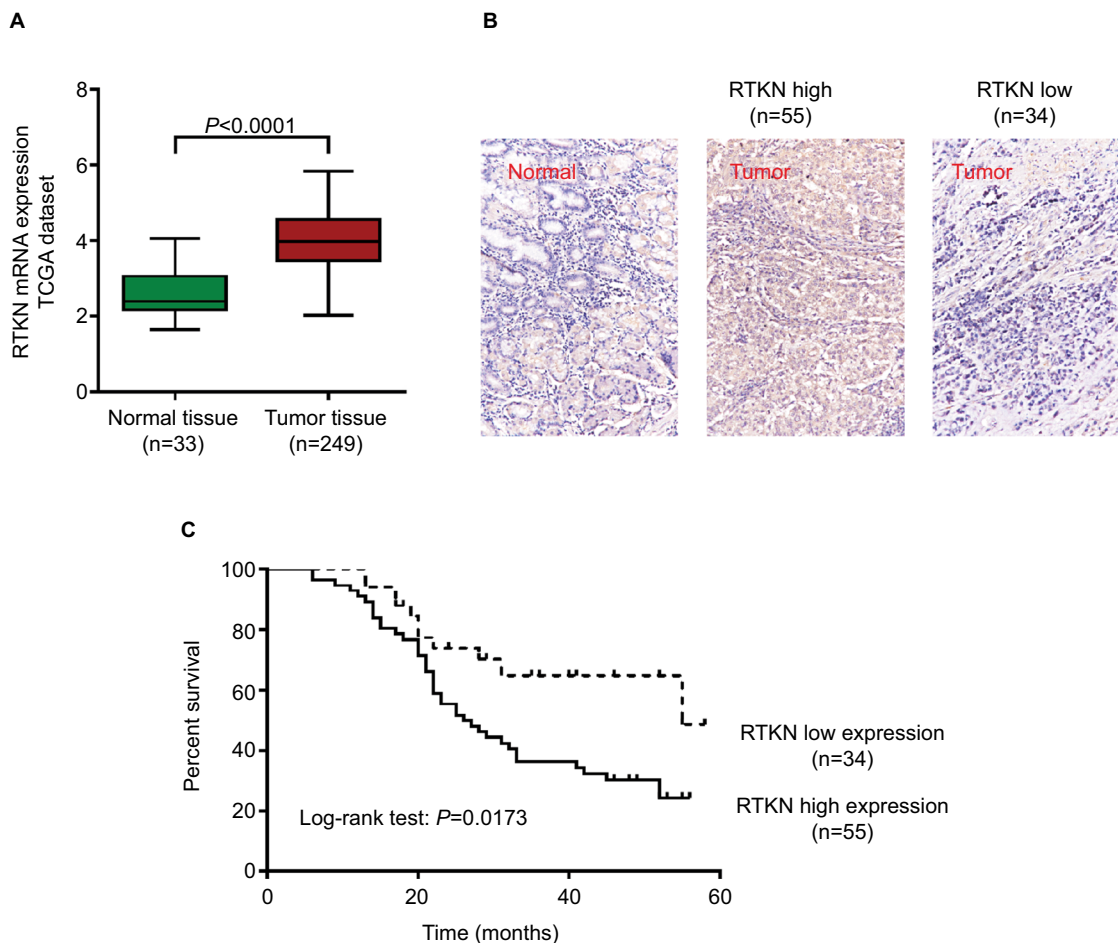

Figure 1 Upregulated RTKN expression correlated with poor prognosis of GC patients.
Notes: (**A**) RTKN expression was significantly increased in GC tissues when compared with the normal tissues based on TCGA-STAD-exp-HiSeq. (**B**) IHC analysis of RTKN protein expression in GC tissues and adjacent normal tissues. Magnification: 200×. (**C**) The overall survival analysis of 89 patients with GC.
Abbreviations: GC, gastric cancer; IHC, immunohistochemistry; TCGA, The Cancer Genome Atlas.

Table 2 The relationship between expression level of RTKN and clinical characteristics in gastric cancer (n=89)

Variables	All cases	RTKN protein		P-value
		Low	High	
Age at surgery (years)				
<55	15	5	40	0.7759
≥55	74	29	45	
Gender				
Male	68	24	44	0.3185
Female	21	10	11	
Tumor size (cm)				
<5	36	19	17	0.0265*
≥5	53	15	38	
TNM				
I + II	45	23	22	0.0162*
III + IV	44	11	33	
Lymph-node metastasis				
Absent	40	23	17	0.0010**
Present	49	11	38	

Notes: *$P<0.05$, **$P<0.01$.

correlated with the poor prognosis of GC patients within 5 years (Figure 1C).

Downregulation of RTKN inhibited GC cell proliferation

Then, we assessed the mRNA and protein expression levels of RTKN in four GC cells – AGS, MKN-45, SGC-7901, and MGC-803 – by real-time PCR and Western blot (Figure S1). Two cell lines, MKN-45 and SGC-7901, showed higher expression. Then, MKN-45 and SGC-7901 cells were transduced with RTKN shRNAs (shRTKN#1, #2, or #3) or control shRNA (shNC). As shown in Figure 2A, B, shNC had no effects on RTKN protein expression when compared with cells without any treatment (Control). RTKN shRNAs obviously reduced RTKN protein expression in both cells, among which shRTKN#1 had the best knockdown efficiency and was selected for the subsequent assays. Real-time PCR results showed a similar trend of RTKN mRNA expression (Figure 2C, D).

The effects of RTKN knockdown on cell growth at 12, 24, 48, and 72 hours were assessed by CCK-8 assay. Results showed that RTKN shRNA had proliferation-inhibiting property in MKN-45 and SGC-7901 cells compared with shNC at 24, 48, and72 hours post transduction (Figure 2E, F).

RTKN shRNA inhibited GC growth in nude mice xenograft model

Next, we determined whether RTKN shRNA could reduce tumor growth in vivo. SGC-7901 cells stably expressing

shNC or shRTKN were injected into athymic nude mice respectively, and tumor volumes were measured for 46 days. The xenografts of shRTKN group grew slower than the shNC tumors in mice (Figure 2G). At 46 days after inoculation, the size and the weight of shRTKN group were less than half of the shNC tumors (Figure 2H, I). These data indicated that downregulation of RTKN inhibited tumor growth in nude mice.

RTKN shRNA arrested G0/G1 cell cycle transition and induced apoptosis in GC cells

To explore how RTKN shRNA inhibited proliferation, we investigated the effects of RTKN on cell cycle and cell apoptosis. The increment of G0/G1-phase cells and the reduction of S-phase cells were observed in MKN-45 and SGC-7901 cells transduced with shRTKN when compared with those transduced with shNC (Figure 3A, B). The protein levels of cell cycle-related proteins, CyclinD1 and PCNA, were also reduced by RTKN shRNA (Figure 3C, D). As indicated by Annexin V-FITC/PI staining assay, knockdown of RTKN significantly induced cell apoptosis compared with shNC (Figure 3E, F). On the contrary, the levels of ATP and lactate dehydrogenase were reduced when RTKN expression was knocked down (Figure S2).

Effects of RTKN shRNA on the p53 pathway

We then performed GSEA to identify RTKN-associated pathways based on TCGA GC dataset. The results showed that KEGG p53 pathway was strongly associated with RTKN expression (Figure 4A).

We further detected the mRNA and protein levels of p53 and several p53 target genes. As illustrated in Figure 4B–E, RTKN shRNA enhanced the transcription and translation of p21, Bax, and PUMA in both GC cell lines, while the total protein and mRNA levels of p53 were not affected by RTKN knockdown. Meanwhile, the p53 acetylation (Lys382 and Ace-p53) was also increased by RTKN shRNA in both GC cell lines (Figure 4D, E).

A previous study has reported that the anti-apoptosis role of RTKN in GC cells is dependent on the activation of NF-κB.[24] The NF-κB activation may be associated with p53 pathway.[32,33] We then overexpressed RTKN and inhibited NF-κB with PDTC in SGC-7901 cells. Results showed that PDTC enhanced the transcription and translation of p21, Bax, and PUMA in cells overexpressing RTKN, suggesting
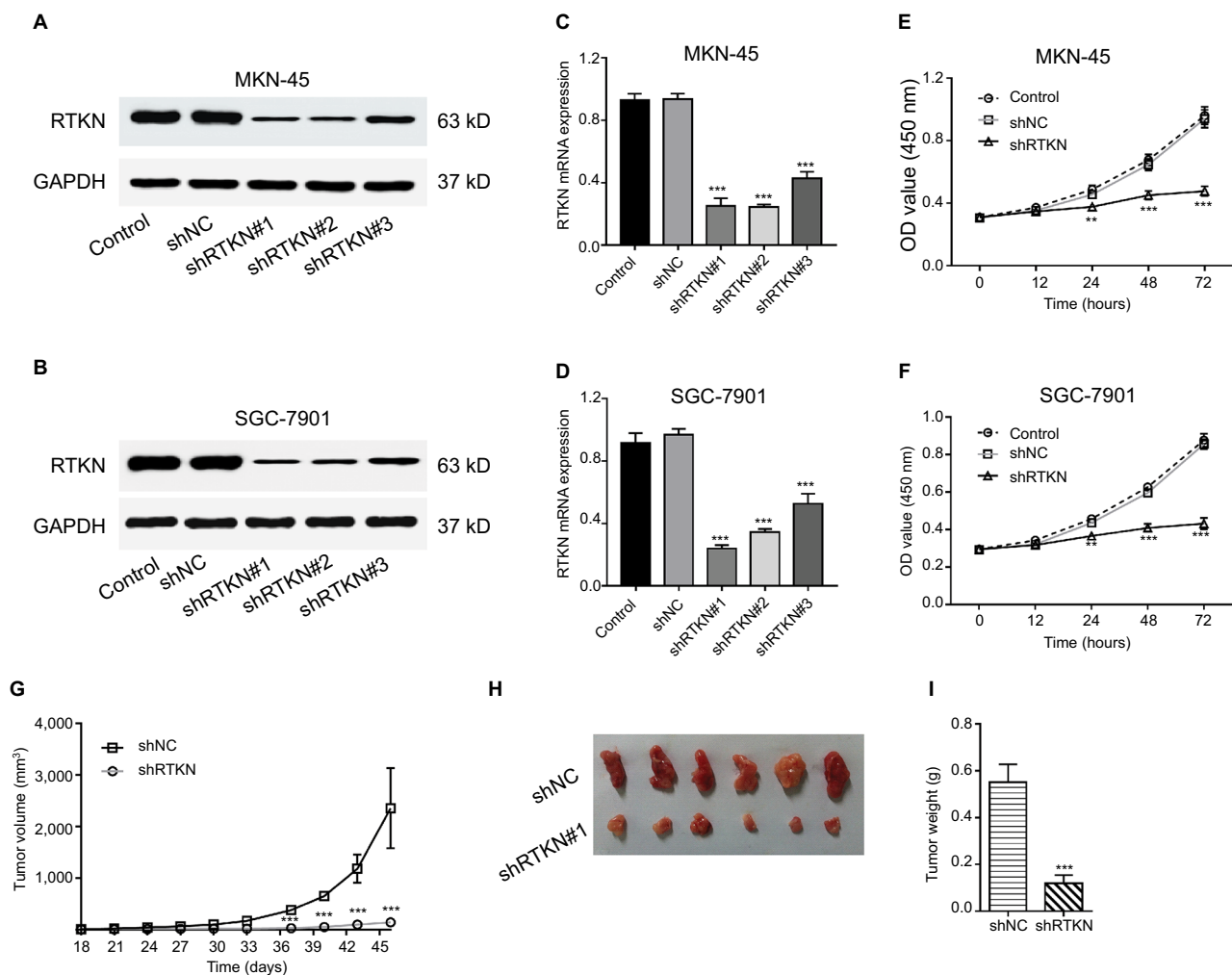
Figure 2 RTKN knockdown inhibited GC cell proliferation in vitro and in vivo.

Notes: (**A–D**) MKN-45 and SGC-7901 cells were transduced with RTKN shRNAs (shRTKN#1, #2, or #3) or control shRNA (shNC) lentivirus. RTKN protein (**A**, **B**) and mRNA (**C**, **D**) levels were evaluated at 48 hours post transduction. Cells without any treatment served as negative control. (**E**, **F**) CCK-8 assay of MKN-45 and SGC-7901 cells transduced with shRTKN#1 (shRTKN) or shNC. In vitro experiments were performed three times independently. Data are presented as mean ± SD. Representative images for Western blotting are shown. (**G–I**) SGC-7901 cells stably transduced with shNC or shRTKN were subcutaneously injected into athymic nude mice (n=6 per group). Tumor diameter and volume (**G**) were evaluated for 46 days. At day 46, mice were sacrificed, and tumors were photographed (**H**) and weighted (**I**). ******$P<0.01$, *******$P<0.001$ vs shNC.

Abbreviations: CCK-8, Cell Counting Kit-8; GC, gastric cancer.

that these genes were also correlated with NF-κB activation (Figure 4F, G).

HDAC1 mediated the effect of RTKN in p53 acetylation, cell cycle arrest, and cell apoptosis

It has been reported that HDAC Class I members (HDAC1, 2, 3, and 8) are able to deacetylate p53 and downregulate the function of p53.[34] GSEA on TCGA dataset showed a positive correlation between the HDAC Class I pathway and RTKN expression in GC tissues (Figure 5A). The mRNA levels of HDAC Class I members were analyzed in MKN-45 and SGC-7901 cells with RTKN knockdown. We found that HDAC1 was decreased the most (Figure 5B, C), and we speculated that HDAC1 was involved in the effects of RTKN on p53 acetylation in GC cells. To test this speculation, we overexpressed RTKN and knocked down HDAC1 in SGC-7901 cells. Results showed that RTKN overexpression enhanced HDAC1 expression, but suppressed the p53 acetylation, while HDAC1 knockdown had reverse effects. RTKN overexpression could not affect the p53 acetylation in HDAC1 knockdown cells (Figure 6A). The alteration of the three p53 target genes was consistent with the changes of the p53 acetylation (Figure 6A, B).
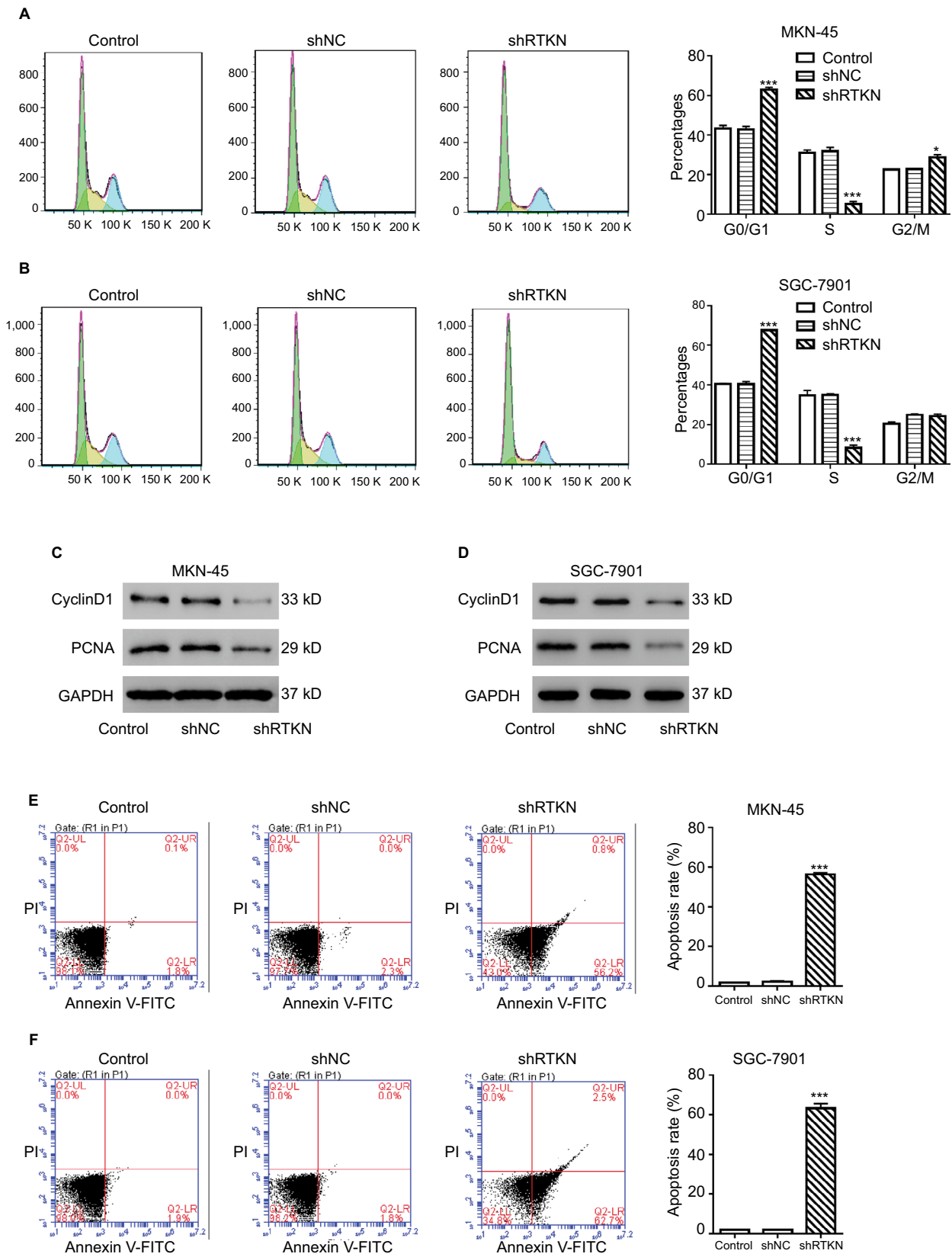
Figure 3 RTKN shRNA arrested G1/S cell cycle transition and induced apoptosis in GC cells.
Notes: MKN-45 and SGC-7901 cells were transduced with shRTKN#1 (shRTKN) or shNC. Cells without any treatment served as negative control. (**A**, **B**) Cells were stained with PI and cell cycle distribution was analyzed by using flow cytometry. (**C**, **D**) Protein levels of CyclinD1 and PCNA were evaluated by Western blotting. (**E**, **F**) Cells were stained with Annexin V-FITC/PI, and apoptotic rates were analyzed by using flow cytometry. Experiments were performed three times independently. Representative images are shown. Data are presented as mean ± SD. *$P<0.05$, ***$P<0.001$ vs shNC.
Abbreviations: FITC, fluorescein isothiocyanate; GC, gastric cancer; PI, propidium iodide.
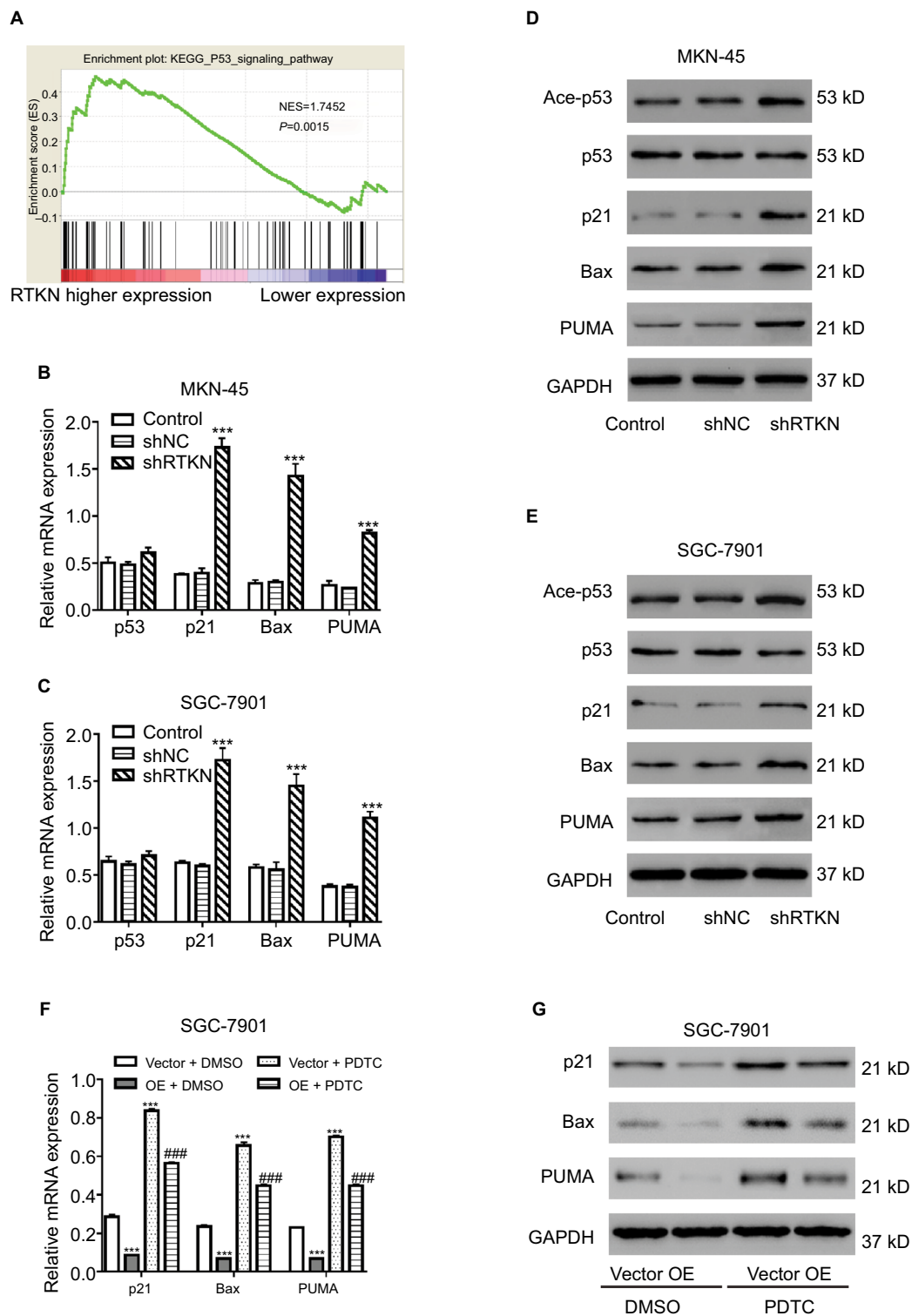
Figure 4 Effects of RTKN shRNA on the p53 pathway.

Notes: (**A**) GSEA was performed using TCGA GC dataset. The p53 pathway was identified with the strongest association with RTKN. (**B**, **C**) mRNA expressions of p53 and three p53 target genes were evaluated by real-time PCR in MKN-45 and SGC-7901 cells transduced with shRTKN#1 (shRTKN) or shNC. ***$P<0.001$ vs shNC. (**D**, **E**) Protein levels of p53, acetylated p53 (Ace-p53), and three p53 target genes were detected by Western blotting. (**F**, **G**) SGC-7901 cells were treated with pLVX-RTKN (OE)/ pLVX-puro (Vector) and 50 μM PDTC (Selleck Chemicals, Houston, TX, USA)/DMSO. mRNA and protein expression of three p53 target genes were detected. ***$P<0.001$ vs Vector + DMSO; ###$P<0.001$ vs OE + DMSO. Experiments were performed three times independently. Data are presented as mean ± SD. Representative images for Western blotting are shown.

Abbreviations: GC, gastric cancer; GSEA, gene set enrichment analysis; PDTC, pyrrolidine dithiocarbamate; TCGA, The Cancer Genome Atlas.
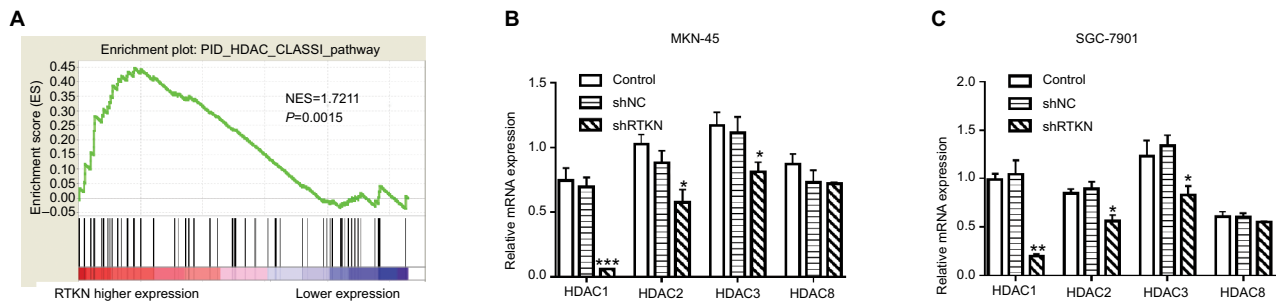
Figure 5 Effects of RTKN shRNA on HDAC1 expression.

Notes: (**A**) GSEA plot showed that the HDAC Class I pathway was strongly associated with RTKN expression on TCGA GC dataset. (**B**, **C**) The mRNA levels of HDAC Class I members in MKN-45 and SGC-7901 cells transduced with shRTKN#1 (shRTKN) or shNC. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs shNC.

Abbreviations: GC, gastric cancer; GSEA, gene set enrichment analysis; HDAC, histone deacetylase; TCGA, The Cancer Genome Atlas.
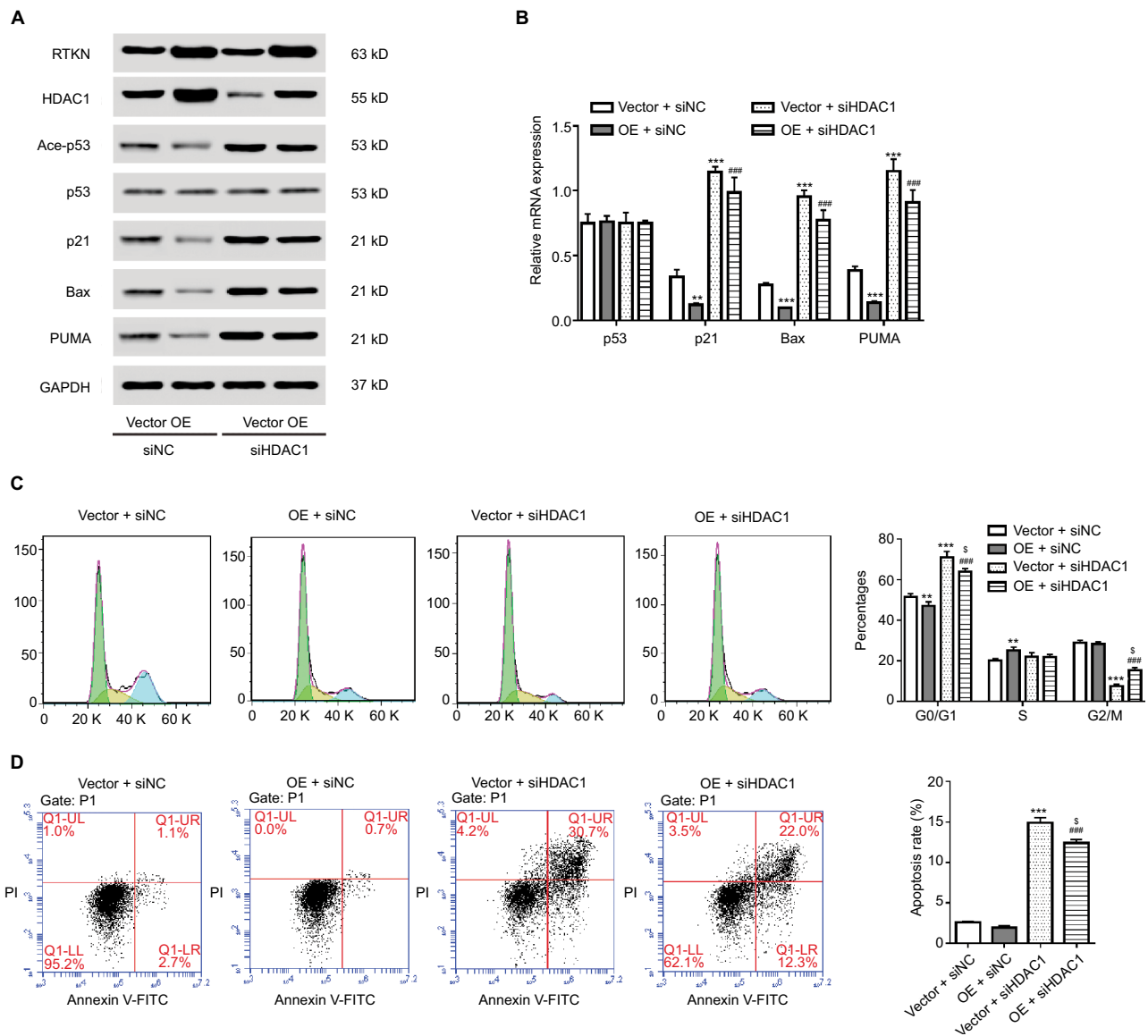
Figure 6 HDAC1 mediated the effect of RTKN in p53 acetylation, cell cycle arrest, and cell apoptosis.

Notes: SGC-7901 cells were treated with pLVX-RTKN (OE)/pLVX-puro (Vector) and HDAC1 siRNA (siHDAC1)/control siRNA (siNC). (**A**) Western blotting analysis was performed and representative images are shown. (**B**) Real-time PCR was conducted to detect the mRNA expression of interested genes. (**C**) Cells were stained with PI and cell cycle distribution was analyzed by using flow cytometry. (**D**) Cells were stained with Annexin V-FITC/PI and apoptotic rates were analyzed by using flow cytometry. Experiments were performed three times independently. **$P<0.01$, ***$P<0.001$ vs Vector + siNC; ###$P<0.001$ vs OE + siNC; $$P<0.05$ vs Vector + siHDAC1.

Abbreviations: FITC, fluorescein isothiocyanate; HDAC, histone deacetylase; PI, propidium iodide.

Results of flow cytometry analyses showed that HDAC1 knockdown arrested G1/S cell cycle transition and induced apoptosis, which was slightly affected when RTKN was simultaneously overexpressed (Figure 6C, D). These data indicated that HDAC1 was involved in the functions of RTKN on p53 acetylation, cell cycle arrest, and cell apoptosis in GC cells.

Discussion

RTKN was initially identified as a putative target for Rho.[14] RTKN is overexpressed in GC[22] and bladder cancer.[23] In the present study, consistent with the previous report,[22] clinical data showed that RTKN was highly expressed in GC tissues based on our own cohort and TCGA dataset. More importantly, we firstly revealed the association between RTKN expression levels and tumor size, TNM stage, lymph-node metastasis, and overall survival. Our findings further supported that RTKN may serve as a useful prognosis marker for GC.

The possible functions of RTKN in cancer cells have been studied. RTKN interacts with S100A4 and contributes to invasive growth of MDA-MB-231 breast cancer cells.[21] RTKN overexpression inhibits GC cell apoptosis, which is dependent on Rho activity and NF-κB activation.[22,24] Here, we found that knockdown of RTKN in GC cells significantly decreased cell proliferation and arrested cells at G0/G1 stage. Further in vivo tumor formation assay suggested that knockdown of RTKN in GC cells hindered the growth of xenografts. In line with the previous studies,[22,24] downregulation of RTKN stimulated cell apoptosis and impaired mitochondrial function targeting cell cycle-related protein, and NF-κB pathway may be applied for cancer therapy.[35–38] Thus, RTKN may serve as a potential therapeutic target for GC.

p53 is a key mediator of cell cycle progression, cell apoptosis, and DNA repair.[25,26] Altered expression and mutation of p53 gene have been reported in various human cancers, such as bladder cancer, hepatocellular carcinoma, and GC.[27–29] Here, GSEA with TCGA dataset identified that RTKN expression was correlated with the p53 signaling pathway. The acetylation levels of p53 correlate with the activation and stabilization of p53.[30] We, therefore, evaluated the effects of RTKN knockdown on the p53 acetylation, and the expression of p53 target genes, p21, Bax, and PUMA. p21, a cyclin-dependent kinase inhibitor, is known to inhibit G1/S transition.[39] Bax and PUMA, two proapoptotic proteins, promote the apoptosis of GC cells.[40] The results showed that the p53 acetylation, and the mRNA and protein levels of all three p53 target genes were obviously increased by RTKN knockdown. On the contrary, RTKN

overexpression in AGS cells, which had a relatively lower level of RTKN, showed opposite effect on the protein levels of p53 acetylation, p21, Bax, and PUMA (Figure S3). Thus, it seems that RTKN performs its biological function through the p53 pathway. The antiapoptosis role of RTKN in GC cells has been suggested to be mediated by NF-κB activation.[24] The association between NF-κB activation and p53 pathway has been studied.[32,33] Here, we found that the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) enhanced the transcription and translation of p21, Bax, and PUMA in cells overexpressing RTKN, suggesting that the p53 pathway was also correlated with NF-κB activation. Our findings further enhance the understanding of the roles and mechanisms of RTKN in gastric tumorigenesis.

Then, we tried to investigate how RTKN regulated the acetylation of p53. HDAC Class I members (HDAC1, 2, 3, and 8) are able to deacetylate p53 and downregulate the function of p53.[34] Here, we revealed a positive correlation between the HDAC Class I pathway and RTKN expression in GC tissues by GSEA. Among the four HDAC Class I members, the most obvious inhibitory effect was found in HDAC1 mRNA expression. We found that RTKN overexpression can hardly rescue the inhibitory influence on cell proliferation, migration, and invasion caused by HDAC1 knockdown. These data suggest that RTKN may serve as a tumor suppressor for GC through regulating HDAC1 expression.

Conclusion

In summary, we demonstrated that RTKN promoted the proliferation through inducing cell cycle progression and inhibiting cell apoptosis in GC cells, and RTKN might exert its biological functions through the HDAC1/p53 pathway. RTKN may serve as an oncogene during gastric tumorigenesis and as a novel target for developing therapeutic approaches to GC treatment, although an in-depth study is required to further validate its therapeutic function.

Acknowledgments

Disclosure

The authors report no conflicts of interest in this work.

References

1. Compare D, Rocco A, Nardone G. Risk factors in gastric cancer. *Eur Rev Med Pharmacol Sci*. 2010;14(4):302–308.
2. Bertuccio P, Chatenoud L, Levi F, et al. Recent patterns in gastric cancer: a global overview. *Int J Cancer*. 2009;125(3):666–673.
3. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin*. 2011;61(2):69–90.
4. Inoue M, Tsugane S. Epidemiology of gastric cancer in Japan. *Postgrad Med J*. 2005;81(957):419–424.
5. Kim HS, Lee H, Jeung HC, et al. Advanced detection of recent changing trends in gastric cancer survival: up-to-date comparison by period analysis. *Jpn J Clin Oncol*. 2011;41(12):1344–1350.
6. Zou XM, Li YL, Wang H, et al. Gastric cancer cell lines induced by trichostatin A. *World J Gastroenterol*. 2008;14(30):4810–4815.
7. Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. *CA Cancer J Clin*. 2014;64(1):9–29.
8. De Angelis R, Sant M, Coleman MP, et al. Cancer survival in Europe 1999–2007 by country and age: results of EUROCARE--5-a population-based study. *Lancet Oncol*. 2014;15(1):23–34.
9. Uemura N, Okamoto S, Yamamoto S, et al. *Helicobacter pylori* infection and the development of gastric cancer. *N Engl J Med*. 2001;345(11):784–789.
10. Kato M, Asaka M. Recent development of gastric cancer prevention. *Jpn J Clin Oncol*. 2012;42(11):987–994.
11. Zabaleta J. Multifactorial etiology of gastric cancer. *Methods Mol Biol*. 2012;863:411–435.
12. Figueiredo C, Garcia-Gonzalez MA, Machado JC. Molecular pathogenesis of gastric cancer. *Helicobacter*. 2013;18(Suppl 1):28–33.
13. Heasman SJ, Ridley AJ. Mammalian Rho GTPases: new insights into their functions from in vivo studies. *Nat Rev Mol Cell Biol*. 2008;9(9):690–701.
14. Reid T, Furuyashiki T, Ishizaki T, et al. Rhotekin, a new putative target for Rho bearing homology to a serine/threonine kinase, PKN, and rhophilin in the rho-binding domain. *J Biol Chem*. 1996;271(23):13556–13560.
15. Ito H, Iwamoto I, Morishita R, et al. Possible role of Rho/Rhotekin signaling in mammalian septin organization. *Oncogene*. 2005;24(47):7064–7072.
16. Sudo K, Ito H, Iwamoto I, Morishita R, Asano T, Nagata K. Identification of a cell polarity-related protein, Lin-7B, as a binding partner for a Rho effector, Rhotekin, and their possible interaction in neurons. *Neurosci Res*. 2006;56(4):347–355.
17. Ito H, Iwamoto I, Morishita R, Nozawa Y, Asano T, Nagata K. Identification of a PDZ protein, PIST, as a binding partner for Rho effector Rhotekin: biochemical and cell-biological characterization of Rhotekin-PIST interaction. *Biochem J*. 2006;397(3):389–398.
18. Wang H, Han M, Whetsell W Jr, et al. Tax-interacting protein 1 coordinates the spatiotemporal activation of Rho GTPases and regulates the infiltrative growth of human glioblastoma. *Oncogene*. 2014;33(12):1558–1569.
19. Reynaud C, Fabre S, Jalinot P. The PDZ protein TIP-1 interacts with the Rho effector rhotekin and is involved in Rho signaling to the serum response element. *J Biol Chem*. 2000;275(43):33962–33968.
20. Nagata K, Ito H, Iwamoto I, Morishita R, Asano T. Interaction of a multi-domain adaptor protein, vinexin, with a Rho-effector, Rhotekin. *Med Mol Morphol*. 2009;42(1):9–15.
21. Chen M, Bresnick AR, O'Connor KL. Coupling S100A4 to Rhotekin alters Rho signaling output in breast cancer cells. *Oncogene*. 2013;32(32):3754–3764.
22. Liu CA, Wang MJ, Chi CW, Wu CW, Chen JY. Overexpression of rho effector rhotekin confers increased survival in gastric adenocarcinoma. *J Biomed Sci*. 2004;11(5):661–670.
23. Fan J, Ma LJ, Xia SJ, et al. Association between clinical characteristics and expression abundance of RTKN gene in human bladder carcinoma tissues from Chinese patients. *J Cancer Res Clin Oncol*. 2005;131(3):157–162.
24. Liu CA, Wang MJ, Chi CW, Wu CW, Chen JY. Rho/Rhotekin-mediated NF-kappaB activation confers resistance to apoptosis. *Oncogene*. 2004;23(54):8731–8742.
25. Farnebo M, Bykov VJ, Wiman KG. The p53 tumor suppressor: a master regulator of diverse cellular processes and therapeutic target in cancer. *Biochem Biophys Res Commun*. 2010;396(1):85–89.
26. Soussi T. The p53 pathway and human cancer. *Br J Surg*. 2005;92(11):1331–1332.
27. Muller PA, Vousden KH. p53 mutations in cancer. *Nat Cell Biol*. 2013;15(1):2–8.
28. Wade M, Li YC, Wahl GM. MDM2, MDMX and p53 in oncogenesis and cancer therapy. *Nat Rev Cancer*. 2013;13(2):83–96.
29. Muller PA, Vousden KH. p53 mutations in cancer. *Nat Cell Biol*. 2013;15(1):2–8.
30. Tang Y, Zhao W, Chen Y, Zhao Y, Gu W. Acetylation is indispensable for p53 activation. *Cell*. 2008;133(4):612–626.
31. Zhang LJ, Lu R, Song YN, et al. Knockdown of anion exchanger 2 suppressed the growth of ovarian cancer cells via mTOR/p70S6K1 signaling. *Sci Rep*. 2017;7(1):6362.
32. Ryan KM, Ernst MK, Rice NR, Vousden KH. Role of NF-kappaB in p53-mediated programmed cell death. *Nature*. 2000;404(6780):892–897.
33. Puszynski K, Bertolusso R, Lipniacki T. Crosstalk between p53 and nuclear factor-B systems: pro- and anti-apoptotic functions of NF-B. *IET Syst Biol*. 2009;3(5):356–367.
34. Juan LJ, Shia WJ, Chen MH, et al. Histone deacetylases specifically down-regulate p53-dependent gene activation. *J Biol Chem*. 2000;275(27):20436–20443.
35. Yang GJ, Zhong HJ, Ko CN, et al. Identification of a rhodium(iii) complex as a Wee1 inhibitor against TP53-mutated triple-negative breast cancer cells. *Chem Commun*. 2018;54(20):2463–2466.
36. Kj W, Zhong HJ, Yang G. Small molecule Pin1 inhibitor blocking NF-κB signaling in prostate cancer cells. *Chem Asian J*. 2018;13(3):275–279.
37. Kim SJ, Lee HW, Baek JH, et al. Activation of nuclear PTEN by inhibition of Notch signaling induces G2/M cell cycle arrest in gastric cancer. *Oncogene*. 2016;35(2):251–260.
38. Wu KJ, Zhong HJ, Li G, et al. Structure-based identification of a NEDD8-activating enzyme inhibitor via drug repurposing. *Eur J Med Chem*. 2018;143:1021–1027.
39. Gartel AL, Radhakrishnan SK. Lost in transcription: p21 repression, mechanisms, and consequences. *Cancer Res*. 2005;65(10):3980–3985.
40. Luo C, Zhu Y, Jiang T, et al. Matrine induced gastric cancer MKN45 cells apoptosis via increasing pro-apoptotic molecules of Bcl-2 family. *Toxicology*. 2007;229(3):245–252.
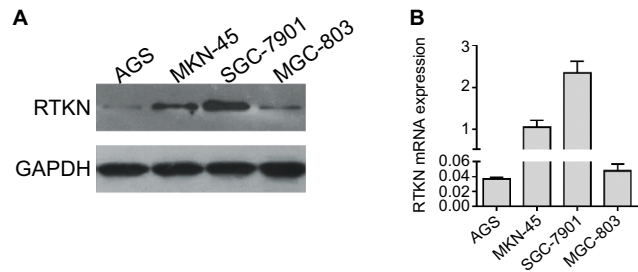
Supplementary materials

A

B

Figure S1 RTKN protein (**A**) and mRNA (**B**) levels were evaluated in four GC cell lines.
Abbreviation: GC, gastric cancer.
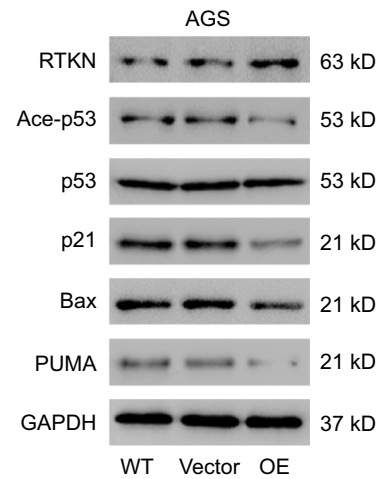
AGS

WT   Vector   OE

Figure S3 Effects of RTKN overexpression on the p53 pathway.
Notes: AGS cells were treated with pLVX-RTKN (OE) or pLVX-puro (Vector). Protein levels of RTKN, p53, acetylated p53 (Ace-p53), and three p53 target genes were detected by Western blotting. Experiments were performed three times independently. Representative images for Western blotting are shown.

A

B

C

D
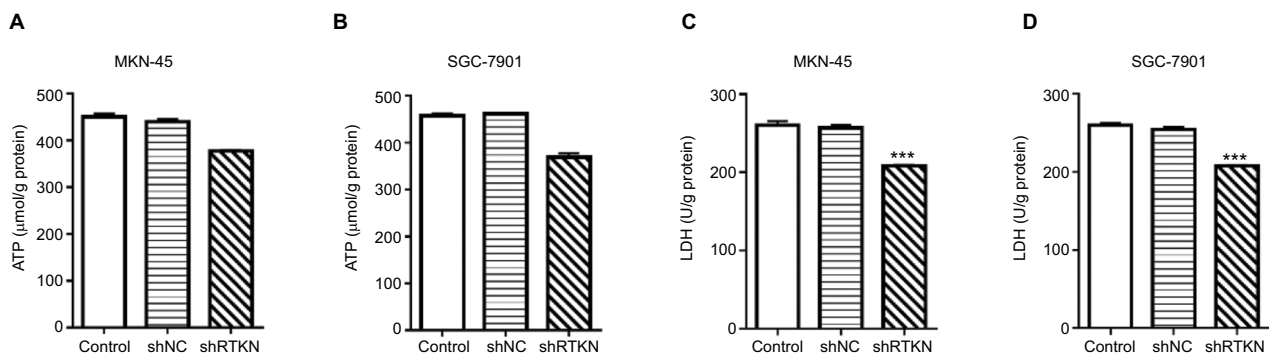
Figure S2 RTKN shRNA decreased ATP content and LDH level in GC cells.
Notes: MKN-45 and SGC-7901 cells were transduced with shRTKN#1 (shRTKN) or shNC. Cells without any treatment served as negative Control. Also, 48 hours later, cell lysate was processed for the measurement of ATP content (**A**, **B**) and LDH (**C**, **D**) by using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's protocols. Experiments were performed three times independently. Data are presented as mean ± SD. ***$P<0.001$ vs shNC.
Abbreviations: GC, gastric cancer; LDH, lactate dehydrogenase.