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Original Article

Downregulation of RECQL4 inhibits gastric cancer cell proliferation and induces cell cycle arrest at G0/G1 phase

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Abstract: Background: The RECQ helicases are a family of DNA repair enzymes that unwind double-stranded nucleic acids and have a central role in maintaining genomic integrity. RECQL4 is a unique member of RecQ helicases family. Although it has been reported to participate in human cancer progression, the role of RECQL4 in gastric cancer (GC) remains unclear. Aims: In this study, we aimed to investigate the function of RECQL4 in GC. Methods: The expression of RECQL4 gene was analyzed by the Oncomine database and GC samples. Overall survival (OS) was performed using Kaplan-Meier plotter online analysis. qRT-PCR, Western blot, MTT assay, clonogenic assay, and flow cytometry were used to analyze the function of RECQL4 in GC. Results: In this study, we found that expression of RECQL4 gene was higher in GC tissues than in the normal tissues by Oncomine database mining and affected patients' OS as analyzed by the Kaplan-Meier plotter online database. RECQL4 was overexpressed in GC samples. Knockdown of RECQL4 significantly suppressed proliferation and colony formation abilities of AGS and SGC-7901 cells. Moreover, cell cycle analysis showed that inhibition of RECQL4 induced cell cycle arrested in G0/G1 phase in AGS and SGC-7901 cells. RECQL4 depletion impaired replication factor loading onto chromatin at the start of S phase. Conclusion: Our study indicates that downregulation of RECQL4 may inhibit GC cell proliferation and induce cell cycle arrest at G0/G1 phase. RECQL4 plays an important role in GC tumorigenesis and may serve as a potential therapeutic target for GC.

Keywords: RECQL4, gastric cancer, cell cycle, proliferation, prognosis

Introduction

Gastric cancer (GC) is the third most frequently diagnosed cancer and third leading cause of cancer death in both men and women in China [1]. According to the cancer statistics in China 2015, the estimated incidence rates for GC are 679.1 per 100,000 population per year, and the mortality rates are about 498.0 per 100,000 population per year [2]. Even though considerable advancements in GC treatment have been made during recent years, the prognosis of GC still remains poor with a 5-year survival rate of approximately 20-40% [3, 4]. Therefore, new treatment strategies for GC are highly needed. Gastric carcinogenesis is a multistep molecular process with sequential activation of oncogenes and inactivation of tumor suppressor genes [5]. Thus, understanding the molecular mechanism of GC is essential not only for devising a better treatment modality but also for developing new therapeutic medicines to improve GC patient survival in the future.

RecQ helicases are a ubiquitous family of DNA unwinding enzymes involved in the maintenance of chromosome stability [6]. RecQ helicase has a unique set of protein-interacting partners, and these interactions dictate its specialized functions in genome maintenance, including DNA repair, recombination, replication, and transcription [7]. Five members of the RecQ family have been found in human cells: RECQL1, WRN, BLM, RECQL4 and RECQL5 [8]. Deficiencies in RecQ helicases can lead to high levels of genomic instability and, in humans, to premature aging and increased susceptibility to cancer [8].

Previous studies have reported the importance of RECQL4 in several tumors. For example, Fang et al. found that overexpression of RecQL4 due to gene amplification play a critical role in human breast tumor progression [9], and Arora et al. demonstrated that shRNA-mediated RecQL4 suppression in MDA-MB453 breast cancer cells significantly inhibited *in vitro* clonogenic survival and *in vivo* tumorigenicity [10]. Su et al. found that overexpression of RECQL4 was positively correlated with the aggressiveness of prostate cancer both *in vitro* and *in vivo*, implying that RECQL4 plays critical role in the carcinogenesis and is a valuable biomarker for prostate cancer [11].

Several studies have reported a relationship between RECQL4 helicases and GC. Kang et al. found that RECQL4 was over amplified in the early stage of GC and may serve as a potential target gene [12]. Recently, Mo et al. reported increased expression of RECQL4 in GC cell lines and primary clinical samples. GC lines with high RECQL4 expression displayed increased resistance to cisplatin treatment because of DNA repair function [13]. Several studies have demonstrated the role of RECQL4 in DNA repair, but its role in DNA replication is still unknown in GC. Targeting DNA Replication Stress may provide an alternative therapeutic approach for cancer [14].

In this study, we observed overexpression of RECQL4 in GC by data mining from the public Oncomine microarray database. The online Kaplan-Meier plotter database was used for analyzing prognosis and proved that overexpression of RECQL4 predicts a poorer prognosis in GC patients. We also clarified the function of RECQL4 in gastric carcinogenesis by knockdown of RECQL4 *in vitro* using a short hairpin RNA (shRNA) lentivirus system and showed that RECQL4 knockdown significantly inhibited cell proliferation and colony formation ability, and led to G0/G1 phase cell-cycle arrest. We found that RECQL4 depletion impairs replication factor loading onto chromatin at the start of S phase and in turn inhibited cell proliferation and induced cell cycle arrest.

Materials and methods

Oncomine database analysis

Expression of RECQL4 gene in GC was examined via the online Oncomine database (www.onocomine.org). Filter combination was applied to analyze corresponding datasets to demonstrate the differences of RECQL4 expression between GC and normal tissues. The data type was defined as mRNA, and the analysis type as cancer vs. normal analysis. Each dataset as revealed by filters was analyzed separately. Differences in RECQL4 expression between different types of GC and normal tissues were compared by using datasets including Cho Gastric [15], DErrico Gastric [16], Chen Gastric [17], and Wang Gastric [18]. The log transformed and normalized expression values of RECQL4 were abstracted, analyzed and read from the scatter plot.

Kaplan-Meier overall survival analysis

We performed Kaplan-Meier survival analysis of RECQL4 using an online tool (http://kmplot.com/analysis/). Here, the database of the Kaplan-Meier plotter was capable to assess the effect of 54,675 genes on survival using 10,188 cancer samples, including breast, lung, ovarian, and GC patients [19]. RECQL4 expression and survival data from Affymetrix microarray, including 1,065 GC patients (ID: 213520_at) were also analyzed. To analyze the prognostic value of RECQL4 gene, the samples were divided into two groups according to the median expression of RECQL4. The two patient groups (high and low expression of RECQL4) were compared via the Kaplan-Meier survival plot. The hazard ratio (HR) with 95% confidence intervals (CI), and the log rank *p* value was computed.

GC tissue samples and immunohistochemical analysis

GC and matched normal gastric tissues from 60 patients who underwent initial surgical resection between August 2008 and January 2009 were selected from the Department of Gastrointestinal Surgery at the Sixth Affiliated Hospital of Sun Yat-sen University, Guangzhou, China. All samples were collected with respective patient's informed consent after approval from the Institute Research Medical Ethics Committee of the Sixth Affiliated Hospital of Sun Yat-sen University. All specimens were previously fixed in 10% buffered formalin and embedded in paraffin wax.

Immunohistochemistry (IHC) staining was performed using a standard Streptavidin-Peroxi-

dase complex method (Beijing Zhongshan God Bridge Company, China). Slides were incubated at 4°C in a moist chamber for overnight with rabbit polyclonal antibody against human RECQL4 (1:25, Abnova, monoclonal antibody (M09), clone 2G8, H00009401-M09). Staining with PBS instead of primary antibody against RECQL4 was used as negative control. The protein expression level of RECQL4 was then evaluated by microscopic examination of stained tissue slides. RECQL4 expression level was determined by integrating the percentage of positive tumor cells and the intensity of positive staining. The intensity of staining was scored as follows: negative (score 0), bordering (score 1), weak (score 2), moderate (score 3), and strong (score 4). We scored the extent of staining according to the percentage of positively stained tumor cells in the field: negative (score 0), 0-25% (score 1), 26-50% (score 2), 51-75% (score 3), and 76-100% (score 4). The product of intensity and extent score was considered as the overall IHC score (values: from 0 to 8). Therefore, RECQL4 expression was thus sorted into 2 categories: high level (grades 4-8) and low level (grades 0-3). RECQL4 staining was scored by two independent pathologists in a blinded manner. For any discrepancy in individual evaluations, the two observers reevaluated the slides together to reach an agreed consensus.

Cell culture

AGS and SGC7091 cells were cultured in RPMI1640 medium supplemented with 10% FBS, 100 units/ml penicillin and 100 mg/ml streptomycin at 37°C and ventilated with 5% CO_2. After 24 hours, the cells were divided into four groups. The cells in the first group were cultured with normal medium and were established as the control group.

Lentiviral vector construction and production

Plasmids were named as pLKO.1-RECQL4-1, pLKO.1-RECQL4-2 and pLKO.1-Scramble were provided by Grady Lab [20]. The sequences were designed as follows: pLKO.1-RECQL4-1: 5'CCGGCGGCTCAACATGAAGCAGAAA CTCGAGTTTCTGCTTCATGTTGAGCCGTTTTTG 3'; pLKO.1-RECQL4-2: 5'CCGGCCTCGATTCCATTATCATTTACTCGAG TAAATGATAATGGAATCGAGG TTTTTG3'; and negative control shRNA, pLKO.1-Scramble: 5'CCGGTCCTAAGGTTAAGTCGCCCTCGCTCGAG CGAGGGCGACTTAACCTTAGG TTTTTG3'. Each DNA was used to transform the E-coli strain DH5-α and purified using plasmid purification kit (Omega, USA). The plasmids were confirmed by DNA sequencing before lentivirus packaging. The vectors containing target sequence or control non-targeting sequence were transfected into HEK293T cells, together with psPAX2 and pMD2.G packaging vectors (Addgene Company, UK) via FuGENE® (Roche company, USA) to generate lentiviruses according to the manufacturer's instructions. After 72 hours, lentiviruses were harvested by purification and precipitation.

Transfection of shRNA targeting RECQL4 gene into GC cells

For lentivirus infection, AGS and SGC-7901cells (40,000 cells/well) were seeded onto six-well plates and transduced with pLKO.1-RECQL4-1, pLKO.1-RECQL4-2, or pLKO.1-Scramble at a multiplicity of infection of 10. Cells were harvested for use in the following assays: Real-time polymerase chain reaction (RT-PCR), Western blot analysis, MTT assay, clonogenic assay, and flow cytometry. Knockdown efficiency of RECQL4 was evaluated by quantitative real-time polymerase chain reaction and Western blot analysis.

qRT-PCR

Total RNA was extracted from GC cells by Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The cDNA was synthesized from total RNA using the following protocol of SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). RECQL4 (assay ID = Hs01548660_g1) and GAPDH probes (assay ID = Hs02758991_g1) were purchased from ABI company. PCR cycle conditions were as follows: 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 2 minutes and 60°C for 1 minute. Fluorescence was analyzed by using the BioRad Connet Real-Time PCR platform (Bio-Rad Laboratories, Inc., Hercules, CA, USA). All samples were examined in triplicate. The mRNA expression levels of RECQL4 were normalized to that of GAPDH mRNA by using comparative threshold cycle (ct) method, in which fold difference was $2^{-\Delta\Delta CT}$.

Western blot analysis

Six days after lentivirus infection, cells were harvested and lysed in ice-cold lysis buffer [50

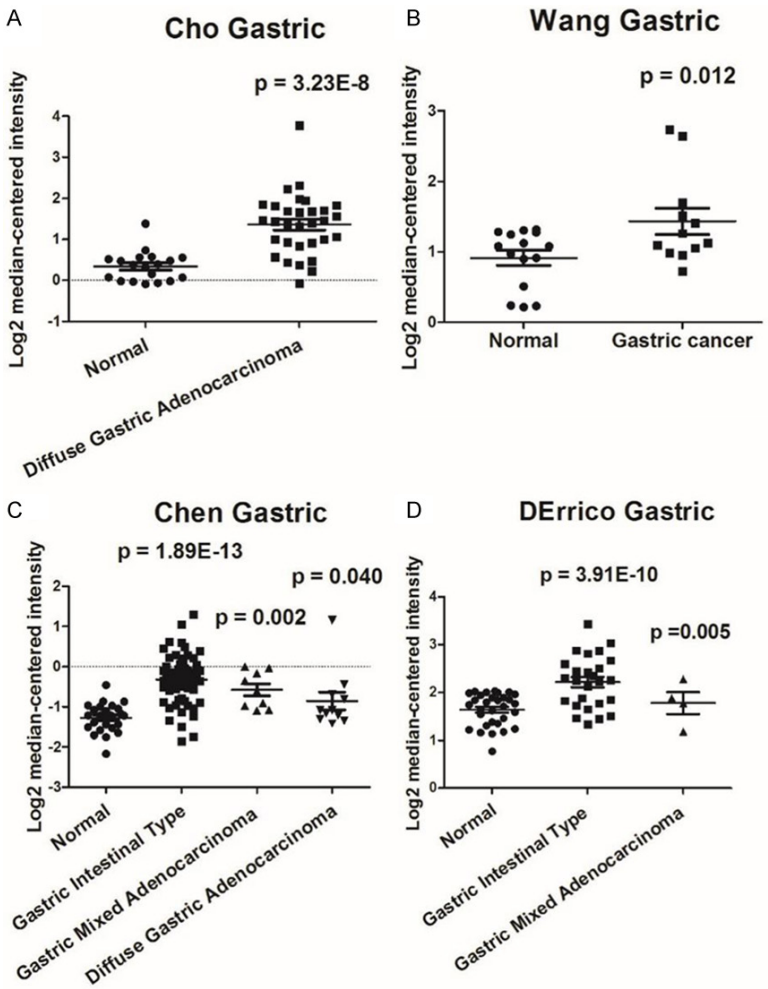

Figure 1. Analysis of RECQL4 mRNA expression in human GC using the Oncomine database. A. Detection of RECQL4 gene expression in normal and diffuse gastric adenocarcinoma tissues by the Cho Gastric dataset. B. Differences in RECQL4 gene expression between normal and gastric cancer tissues are shown by the Wang Gastric dataset. C. RECQL4 expression in the normal, gastric intestinal type, gastric mixed adenocarcinoma and diffuse gastric adenocarcinoma tissues as shown by the Chen Gastric dataset. D. Differences in RECQL4 expression among normal tissues, gastric intestinal type and gastric mixed adenocarcinoma are shown by the DErrico Gastric dataset. *P* values were calculated by using two-tailed and Student's t test.

mM Tris, 2% sodium dodecyl sulphate (SDS), 5% glycerinum, 100 mM NaCl, 1 mM ethylene diamine tetra acetic acid, pH 6.8]. Protein concentrations of the lysate were determined using BCA Protein Assay Kit (Sangon Biotech, China). A total of 50 μg protein in each lane was electrophoresed on 10% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membrane (BioRad), and incubated with rabbit anti-RECQL4 (1:100; Abnova, Taiwan), and GAPDH (1:5000, Santa Cruz Biotechnology) for overnight at 4°C. Membranes were then incubated with HRP-conjugated Donkey Anti-Rabbit IgG (1:5000; Sangon Biotech, China). Signals were detected using ECL luminescence reagent (Sangon Biotech, China) according to the manufacturer's protocol. GAPDH was used as the internal standard. Antibodies were as follows: Polyclonal antibodies against RECQ1 (BL2074) and WRN (NB100-471) were purchased from Bethyl Laboratories and Novus Biologicals, respectively. Anti-Orc2 (3B7) antibodies were from MBL. MCM3 (N-19) and PCNA (F-2) antibodies were purchased from Santa Cruz Biotechnologies. RPA (BL915) antibody was purchased from Bethyl Laboratories. Anti-α-tubulin (B-5-1-2) antibody was obtained from Sigma.

MTT assay

The effect of RECQL4 on cell viability was analyzed using MTT Cell Proliferation and Cytotoxicity Assay Kit (Sangon Biotech, China) based on growth curves of AGS and SGC7901 cells *in vitro*. Briefly, cells were seeded in 96-well plates at a concentration of 2000 cells/well in 100 μl/well after 3 days of lentivirus infection. Cells were then further cultured in this manner for 1-5 days. Four hours before the termination of culture, MTT reagent (5 mg/ml) was added at a volume of 10 μl/well. The cells were then gently mixed and incubated for 4 hours in a CO_2 incubator. Afterwards, the Formazan Solubilization Solution was added at a volume of 100 μl/well and mixed gently for 10 minutes to dissolve the formazan crystals. The absorbance at 570 nm of each well was determined using the Multiskan Spectrum microplate reader (Thermo Scientific, USA).

Clonogenic assay

After 3 days of lentivirus infection, AGS and SGC7901 cells (500 cells/well) were seeded in

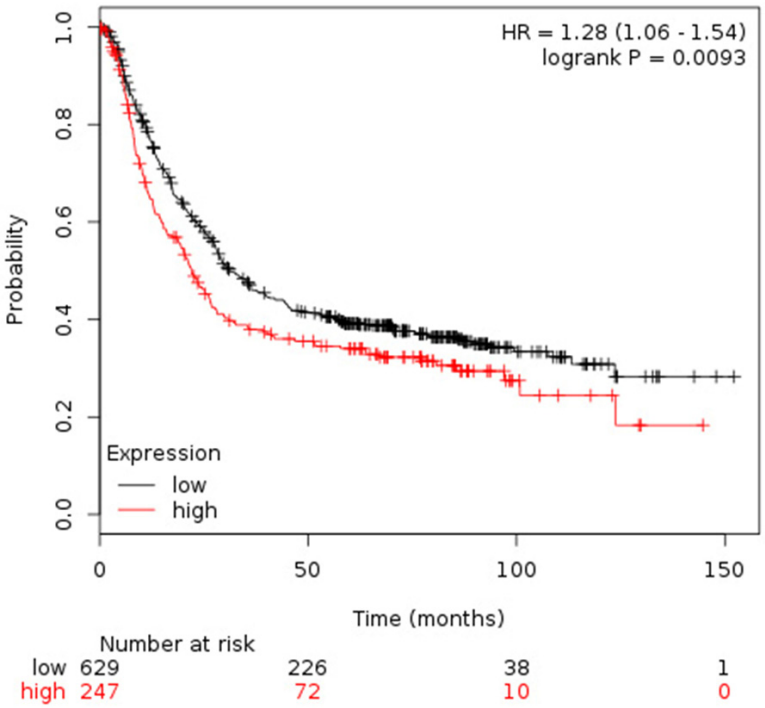

Figure 2. Correlation of RECQL4 mRNA expression with overall survival in gastric cancer patients. Kaplan-Meier plot analysis for overall survival in gastric cancer patients according to the expression levels of RECQL4. HR: hazard ratio; CI: confidence interval; *P* value: log-rank test.

six-well plates and incubated for 10 days to form normal colonies. The media was replaced every 3 days. Then the cells were fixed with paraformaldehyde for 30 min at room temperature. The fixed cells were washed twice with phosphate buffered saline (PBS), stained with 1% crystal violet (Sangon Biotech, China) for 10 min, washed with ddH_2O, and then air dried. The total number of colonies that contain > 50 cells was counted under a light microscope.

Flow cytometry for cell cycle analysis

After 4 days of lentivirus infection, AGS and SGC7901 cells were seeded in 6-cm dishes at a density of 80,000 cells/dish and cultured for 48 hours. Cells were then released by digestion with trypsin and harvested. After centrifugation, the cell pellet was washed twice with pre-cooled PBS and fixed with precooled 70% ethanol for overnight at 4°C. The cells were then washed twice with pre-cooled PBS and resuspended in PBS containing 10% FBS, and stained with propidium iodide (P4170; SIGMA) solution (PI, 50 μg/ml, 100 μg/ml RNase in PBS) at 37°C in the dark for 30 min. Finally, the cells were analyzed for cell cycle phase by using FACS can (Beckman Coulter, Brea, USA). Percentage of cells at various phases of the cell cycle was analyzed using the FlowJo software.

Statistical analysis

The results are expressed as mean ± SD. ANOVA was used to assess the differences among the groups and Post hoc test was used to assess the differences between the two groups. $P < 0.05$ was defined as a significant difference. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, USA).

Results

RECQL4 mRNA was overexpressed and predicted poor prognosis in GC

By using the Oncomine database mining, we examined and analyzed the expression level of RECQL4 in GC tissues. As shown in **Figure 1**, expression of RECQL4 was significantly elevated in GC tissues (n = 19, P = 3.23E-8) compared with the normal tissues (n = 31) by using the Cho Gastric dataset (**Figure 1A**). RECQL4 expression was also significantly increased in the Wang Gastric dataset (n = 27, P = 0.012, **Figure 1B**). The Chen Gastric dataset showed RECQL4 expression in gastric intestinal type adenocarcinoma (n = 63, P = 1.89E-13), gastric mixed adenocarcinoma (n = 8, P = 0.002), and diffuse gastric adenocarcinoma (n = 12, P = 0.040) was higher than that in the normal tissues (n = 26, P = 0.012, **Figure 1C**). Another independent DErrico Gastric dataset revealed that RECQL4 was upregulated in gastric intestinal type adenocarcinoma (n = 26, P = 3.91E-10) and gastric mixed adenocarcinoma (n = 4, P = 0.005) compared with normal colon tissues (**Figure 1D**).

The correlation between RECQL4 mRNA expression levels and overall survival (OS) was analyzed in GC patients using the Kaplan-Meier plotter online software based on a public database. OS of patients with low expression of

Table 1. Association of RECQL4 expression with the clinicopathological characteristics of GC

Characteristics	Category	Total	RECQL4 expression		*P* value
			Low expression (n = 33, 55%)	High expression (n = 27, 45%)	
Gender	Male	39	24 (61.5%)	15 (38.5%)	0.165
	Female	21	9 (42.9%)	12 (57.1%)	
Age (yr)	≥ 60	37	20 (54.05)	17 (45.95)	0.706
	< 60	23	13 (56.52)	10 (43.48)	
Tumor Location	U	21	13 (61.9%)	8 (38.1%)	0.430
	M, L	39	20 (51.3%)	19 (48.7%)	
Tumor Size (cm)	≥ 5	15	9 (60.0%)	6 (40.0%)	0.653
	< 5	45	24 (53.3%)	21 (46.7%)	
Histological differentiated	Well, Moderate	15	7 (46.7%)	8 (53.3%)	0.454
	Poorly, others	45	26 (57.8%)	19 (42.2%)	
Depth of invasion	T1-T2	`18	6 (33.3%)	12 (66.7%)	0.035
	T3-T4	43	27 (62.8%)	16 (37.2%)	
Lymph node metastases	Absent	19	8 (42.1%)	11 (57.9%)	0.172
	Present	41	25 (61.0%)	16 (39.0%)	
Distant metastasis	Absent	47	26 (55.3%)	21 (44.7%)	0.925
	Present	13	7 (53.8%)	6 (46.2%)	

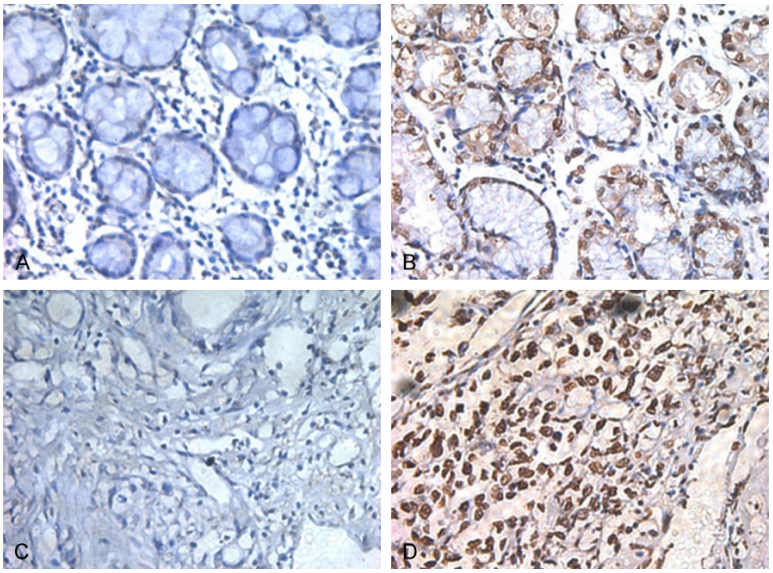

Figure 3. Immunohistochemical examination. Immunohistochemical staining of RECQL4 in the (A) Normal gastric mucosal tissues with low expression of RECQL4, 200X; (B) Normal gastric mucosal tissues with high expression of RECQL4, 200X; (C) Gastric cancer with low expression of RECQL4, 200X; (D) Gastric cancer with high expression of RECQL4, 200X.

RECQL4 was remarkably longer than those patients with high expression (HR = 1.28, 95% CI = 1.06-1.54, P = 0.0093, **Figure 2**).

RECQL4 was overexpressed in GC samples

To verify the above predictions, GC samples and matched normal gastric tissues from 60 patients were selected. The patients' information is presented in **Table 1**. Immunohistochemical analysis showed that RECQL4 positivity was clearly localized in the nuclei of GC cells and few in the cytoplasm (**Figure 3**). The positive rate of RECQL4 in the GC samples was 55% (33/60), which was significantly higher than that of the normal gastric mucosa specimens, 23.3% (14/60), (P < 0.05).

Taken together, our findings indicate that high expression of RECQL4 plays a potential role in gastric carcinogenesis.

RECQL4 expression is reduced in lentivirus-transduced GC cells

To determine the function of RECQL4 in GC cells, the RECQL4 gene was knocked down using a lentivirus-delivered shRNA that specifically targets human RECQL4 [pLKO.1-RECQL4-1 & pLKO.1-RECQL4-2], (**Figure 4A**, **4D**). RECQL4 mRNA levels in the groups transferred with pLKO.1-RECQL4-1 group and pLKO.1-RECQL4-2 group were significantly lower than those in

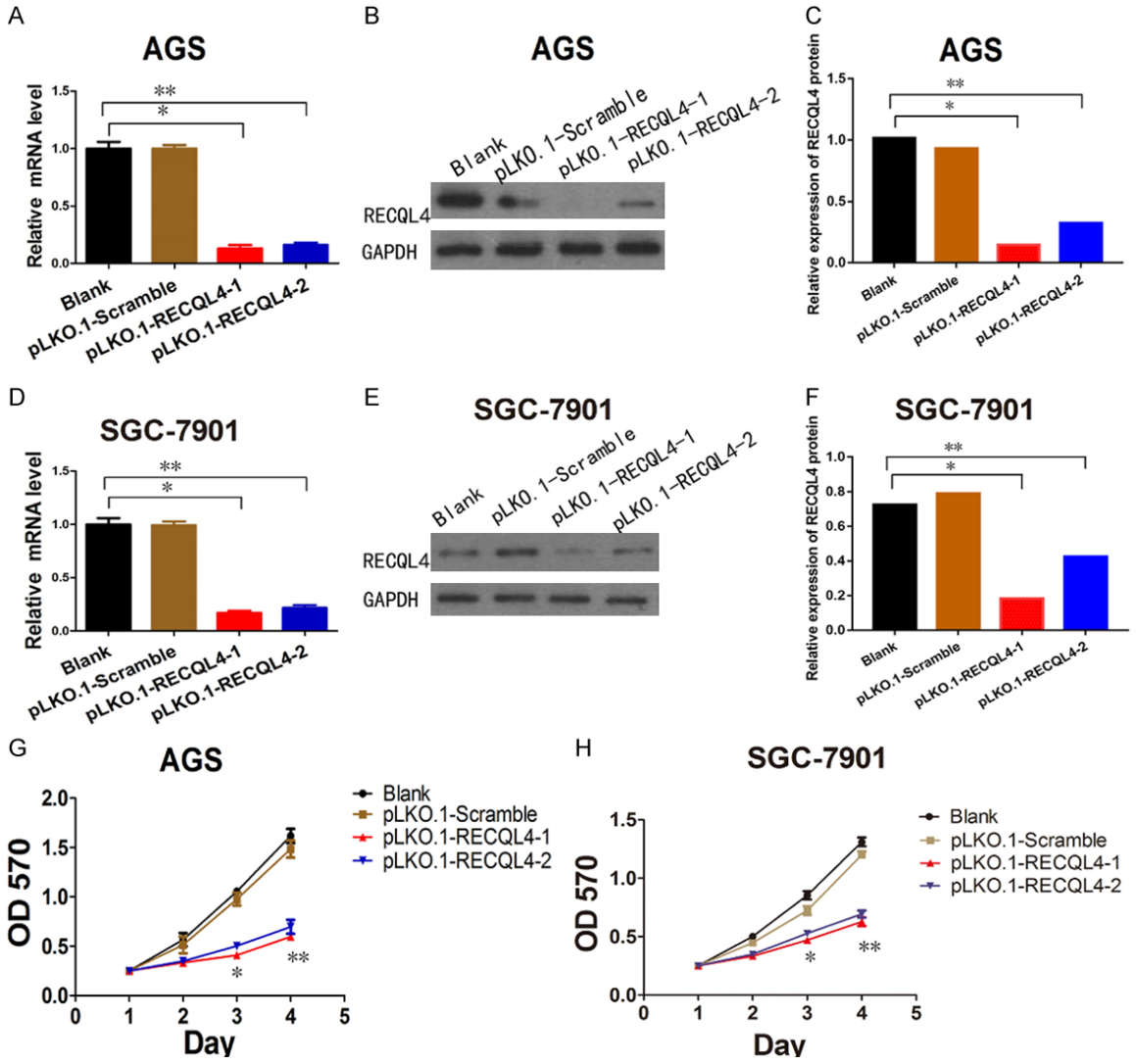

Figure 4. Lentivirus-mediated shRNA specifically reduced RECQL4 expression and inhibited proliferation of AGS cells and SGC-7901 cells. A. qRT-PCR analysis of RECQL4 mRNA levels in AGS cells after lentivirus infection, *P < 0.01, **P < 0.05. B. Western blot analysis of RECQL4 protein levels in AGS cells after lentivirus infection. C. Quantification of Western blot analysis of RECQL4 protein levels, normalized to levels of GAPDH; *P < 0.01, **P < 0.05, compared to Blank group. D. qRT-PCR analysis of RECQL4 mRNA levels in SGC-7901 cells after lentivirus infection, *P < 0.01, **P < 0.05. E. Western blot analysis of RECQL4 protein levels in SGC-7901 cells after lentivirus infection. F. Quantification of Western blot analysis of RECQL4 protein levels, normalized to levels of GAPDH; *P < 0.01, **P < 0.05, compared to the Blank group. G. Growth curve of AGS cells with pLKO.1-Scramble, pLKO.1-RECQL4-1 and pLKO.1-RECQL4-2 treatment was assessed by MTT assay. H. Growth curve of SGC-7901 cells with pLKO.1-Scramble, pLKO.1-RECQL4-1 and pLKO.1-RECQL4-2 treatment was assessed by MTT assay. Blank: No treatment. *P < 0.05, **P < 0.001, compared to the Blank group.

the pLKO.1-Scramble group. The knockdown efficacy of RECQL4 by pLKO.1-RECQL4-1 and pLKO.1-RECQL4-12 was 90% and 85.4% in AGS cells and 86.5% and 80.9% in SGC-7901 cells, suggesting that shRNA knockdown was specific and the loss-of-functional outcome was not possibly caused by off-target effects (**Figure 4A**, **4D**). Western blot analysis also showed that RECQL4 protein was reduced following the RECQL4 knockdown (**Figure 4B**, **4C**, **4E**, **4F**). These results suggested that RECQL4 shRNA could significantly downregulate RECQL4 expression in GC cells.

Knockdown of RECQL4 inhibits proliferation of GC cells

MTT test showed that the OD value that represented cell proliferation rate was increased from 0.25 to 1.8 in the control group and 0.25

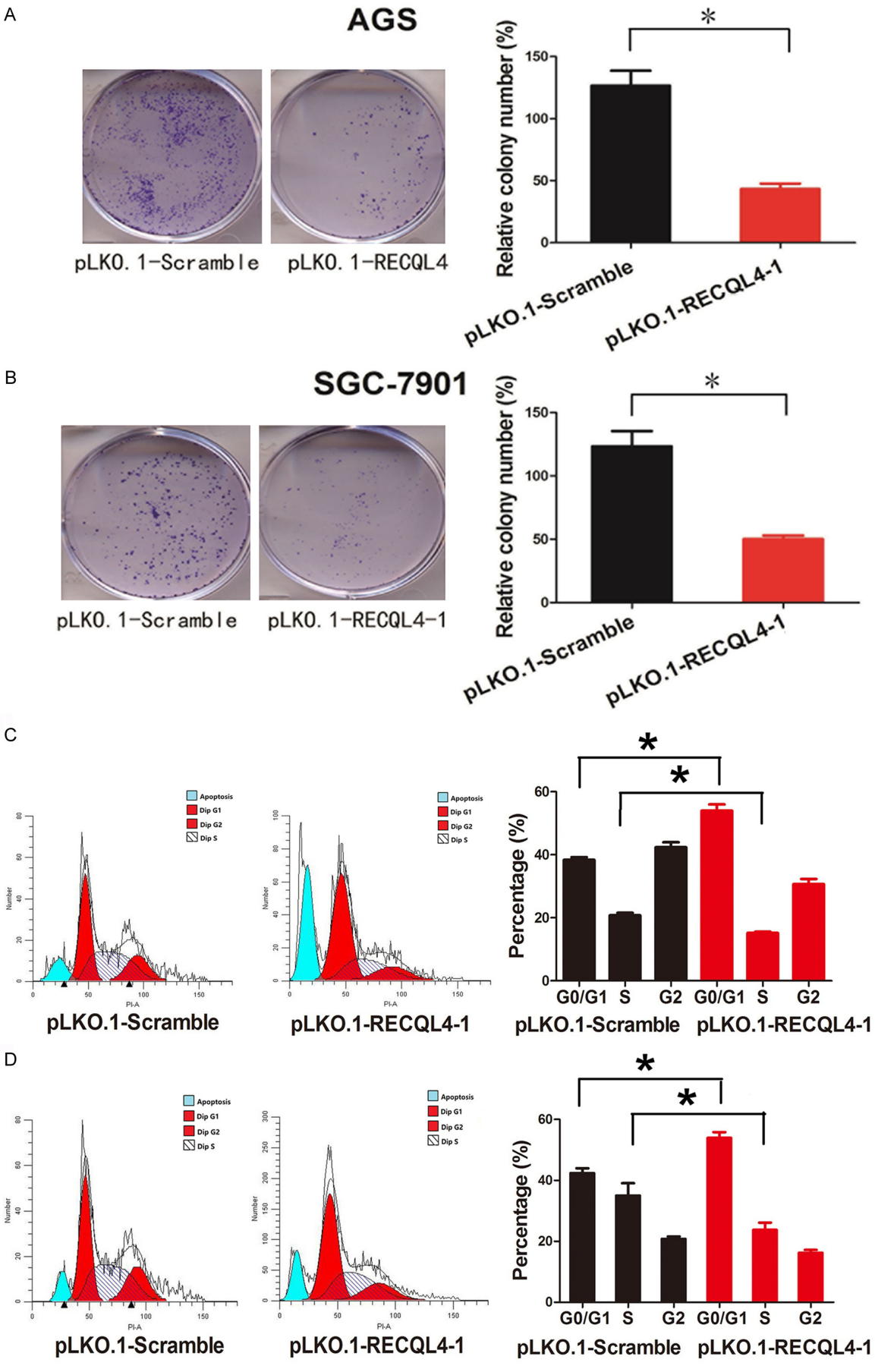
A
AGS
pLKO. 1-Scramble
pLKO. 1-RECQL4
Relative colony number (%)
150
100
50
0
*
pLKO.1-Scramble
pLKO.1-RECQL4-1
B
SGC-7901
pLKO. 1-Scramble
pLKO. 1-RECQL4-1
Relative colony number (%)
150
100
50
0
*
pLKO.1-Scramble
pLKO.1-RECQL4-1
C
Apoptosis
Dip G1
Dip G2
Dip S
Number
PI-A
pLKO.1-Scramble
pLKO.1-RECQL4-1
Percentage (%)
60
40
20
0
*
*
G0/G1
S
G2
G0/G1
S
G2
pLKO.1-Scramble
pLKO.1-RECQL4-1
D
Apoptosis
Dip G1
Dip G2
Dip S
Number
PI-A
pLKO.1-Scramble
pLKO.1-RECQL4-1
Percentage (%)
60
40
20
0
*
*
G0/G1
S
G2
G0/G1
S
G2
pLKO.1-Scramble
pLKO.1-RECQL4-1

Figure 5. Knockdown of RECQL4 inhibited the number of colonies formed and induced cell cycle arrest in AGS cells and SGC-7901 cells. A. Number of colonies formed in AGS cells treated with pLKO.1-Scramble and pLKO.1-RECQL4-1. B. Number of colonies formed in SGC-7901 cells treated with pLKO.1-Scramble and pLKO.1-RECQL4-1. C. Analysis of cell cycle distribution and representative graphs of two independent experiments regarding the percentage of cells in different phases of AGS cells. D. Analysis of cell cycle distribution and representative graphs of two independent experiments about the percentage of cells in different phases in SGC-7901 cells. *: $P < 0.001$.

to 1.7 pLKO.1-Scramble group within 5 days after lentivirus transduction, while cell proliferation in the pLKO.1-RECQL4-1 & pLKO.1-RECQL4-2 groups was relatively slow, increasing from 0.25 to 0.8 in the pLKO.1-RECQL4-1 group and from 0.25 to 0.85 in the pLKO.1-RECQL4-2 group ($P < 0.001$; **Figure 4G**, **4H**) respectively. These results indicate that proliferation of GC cells is significantly impeded following RECQL4 knockdown. This is because the GC cells in pLKO.1-RECQL4-1 group were slow, and so we chose this group for further studies.

Knockdown of RECQL4 restrains colony formation of GC cells

In addition, the colony formation capacity of GC cells was examined after RECQL4 knockdown. Representative photographs of the number of cells per colony and the number of colonies per well are shown in **Figure 5A**, **5B**. Both cell and colony numbers were much smaller in the pLKO.1-RECQL4-1 groups than those in the pLKO.1-Scramble. In AGS cells, there were (129 ± 5) colonies in pLKO.1-Scramble vs. (78 ± 2) colonies in pLKO.1-RECQL4-1 group, indicating that the colony number was reduced by nearly 40% ($P < 0.005$). In SGC-7901 cells, the colony number (52 ± 8) was reduced by 50% in pLKO.1-RECQL4-1 group compared with that of pLKO.1-Scramble (119 ± 7), ($P < 0.001$).

Knockdown of RECQL4 arrests cell cycle progression of GC cells

To investigate whether cell cycle arrest contributed to cell growth inhibition, the cell cycle distribution in GC cells was analyzed using fluorescence-activated cell sorting (FACS). Results showed that knockdown of RECQL4 in AGS increased the percentage of G0/G1-phase cells using the pLKO.1-RECQL4-1 vector (54.50% ± 0.30% vs. 39.95% ± 0.13%; $P < 0.001$) and decreased the percentage in S phase cells (30.17% ± 0.52% vs. 43.59% ± 0.20%; $P < 0.001$) compared to the pLKO.1-Scramble (**Figure 5C**, **5D**). Knockdown of RECQL4 in SGC-7901 increased the percentage of G0/G1-phase cells using the pLKO.1-RECQL4-1 vector (58.30% ± 0.18% vs. 44.29% ± 0.09%; $P < 0.001$) and decreased the percentage of S phase cells (22.72% ± 0.25% vs. 29.85% ± 0.23%; $P < 0.001$) compared to the pLKO.1-Scramble (**Figure 5C**, **5D**).

RECQL4 depletion impairs replication factor loading onto chromatin at the start of S phase

In order to investigate the possible mechanistic role, we examined the order of chromatin loading of replication factors in RECQ4-depleted cells at the onset of S phase. Cells were synchronized by serum starvation during G0 prior to release by the addition of serum and concurrent transfection with pLKO.1-RECQL4-1 vector. Cells were harvested 24 hours after serum stimulation and transfected in order to isolate RECQ-depleted cell populations that were highly enriched in S phase. Using this protocol, we achieved 90% RECQ4 depletion (**Figure 6**). Our results demonstrate that RECQ4 depletion does not affect chromatin recruitment of MCM3 or ORC2. This indicates that RECQ4 is loaded on the origins after assembly of pre-replication complex (**Figure 6**). In contrast, recruitment of PCNA to chromatin was decreased markedly in RECQ4-depleted-cells, while RPA loading was suppressed only in RECQ4-depleted cells (**Figure 6**). These results indicate that RECQ4 is loaded onto the origins before origin firing. Depletion of RECQL4 may inhibit the cell cycle by blocking origin firing.

Discussion

Currently, Oncomine is the biggest cancer microarray database that is available. In the present study, we used data mining of our independent microarray datasets (Cho Gastric, DErrico Gastric, Chen Gastric, Wang Gastric and Cui Gastric) in the Oncomine database and demonstrated the overexpression of RECQL4 in GC. Online Kaplan-Meier plotter analysis proved that RECQL4 predicts a poorer prognosis rate in GC patients. Similar correlation has also been reported between RECQL4 expression

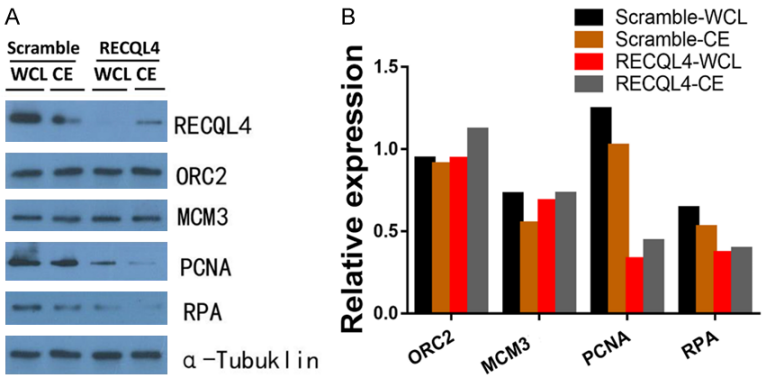

Figure 6. RECQL4 depletion impairs replication factor loading onto chromatin at the start of S phase. A. Western blot analysis of whole and fractionated cell extracts from synchronized AGS cells. The subcellular distribution of RECQL4 and four additional replication proteins, together with α-tubulin control, in whole-cell lysates (WCL) and chromatin-enriched (CE) fractions were prepared from RECQL4-, or control (Scramble)-depleted cells. B. Quantification of Western blot analysis of four additional replication proteins, normalized to levels of α-tubulin.

and other types of cancers such as colorectal [20] and breast cancers [9]. Our results show that RECQL4 protein was overexpressed significantly in human GC samples. These results implied that RECQL4 plays a potential role in carcinogenesis and RECQL4 may serve as a novel biomarker for predicting the prognosis in GC.

Although the experimental and molecular details are not yet clearly understood, specific roles of RECQL4 in DNA replication have been postulated [7]. RECQL4 plays a role during initiation of DNA replication in Drosophila and chicken DT40 cells [21-23], whereas it is associated with replication origins during G1/S phase and interacts with replisome factors MCM10, MCM2-7 helicase, CDC45, and GINS [24, 25]. In eukaryotes, the initiation of DNA replication is a multistep process that requires assembly of pre-replicative complex (pre-RC), activation of pre-RC, and formation of the replisome [26]. pre-RC assembly occurs only in G1 phase [26]. RecQL4 plays an essential role in the assembly of CMG complex [27]. Proliferation of cancer cells might be inhibited when the DNA replication-initiation proteins were silenced [28]. CMG complex formation was significantly inhibited when RecQL4 was depleted in HeLa cells [25]. In this study, knockdown of RECQL4 inhibited proliferation of GC cells and arrested the cells in G0/G1 phase. In addition, cell colony formation was significantly inhibited when RecQL4 was depleted in AGS and SGC-7901 cells. Furthermore, our results demonstrate that RECQ4 depletion does not affect chromatin recruitment of MCM3 or ORC2. In contrast, the recruitment of PCNA and RPA to chromatin was decreased markedly in RECQ4-depleted-cells. These results indicate that depletion of RECQL4 may inhibit the cell cycle by blocking origin firing. These data suggest that the function of RECQL4 was particularly relevant for firing of dormant origins after DNA replication stress that either slows or stalls normal replication forks. This function of RECQ4 may play a role during the early S phase to stabilize or repair replication forks leading to the cell cycle arrest at G0/G1 phase and inhibit the proliferation of GC cells. These findings suggest that RECQL4 may act as a potential therapeutic target for patients with GC.

In conclusion, RECQL4 is a novel regulator of cell proliferation in GC. Although the precise underlying mechanism regarding the effect of RECQL4 on GC cell proliferation still needs to be further elucidated, this study provides a basic concept for recognizing a novel target molecule that may control the development and progression of human GC. Further research is required to provide more convincing evidence to support the possibility of using RECQL4 as a new and potential therapeutic target against GC.

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Disclosure of conflict of interest

None.

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