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RP11-789C1.1 inhibits gastric cancer cell proliferation and accelerates apoptosis via the ATR/CHK1 signaling pathway

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Abstract

Background: Long non-coding RNAs (lncRNAs) plays an important role in the progression of gastric cancer (GC). Their involvement ranges from genetic regulation to cancer progression. However, the mechanistic roles of RP11-789C1.1 in GC are not fully understood.

Methods: We identified the expression of lncRNA RP11-789C1.1 in GC tissues and cell lines by real-time fluorescent quantitative polymerase chain reaction. A series of functional experiments revealed the effect of RP11-789C1.1 on the proliferation of GC cells. *In vivo* experiments verified the effect of RP11-789C1.1 on the biological behavior of a GC cell line. RNA pull-down unveiled RP11-789C1.1 interacting proteins. Western blot analysis indicated the downstream pathway changes of RP11-789C1.1, and an oxaliplatin dosing experiment disclosed the influence of RP11-789C1.1 on the drug sensitivity of oxaliplatin.

Results: Our results demonstrated that RP11-789C1.1 inhibited the proliferation of GC cells and promoted the apoptosis of GC cells. Mechanistically, RP11-789C1.1 inhibited checkpoint kinase 1 (CHK1) phosphorylation by binding ataxia-telangiectasia mutated and Rad3 related (ATR), a serine/threonine-specific protein kinase, promoted GC apoptosis, and mediated oxaliplatin sensitivity.

Conclusion: In general, we discovered a tumor suppressor molecule RP11-789C1.1 and confirmed its mechanism of action, providing a theoretical basis for targeted GC therapy.

Keywords: Long non-coding RNA; RP11-789C1.1; Stomach neoplasms; ATR; CHK1; Oxaliplatin

Introduction

Gastric cancer (GC) is one of the most serious malignant gastrointestinal tumors and the third leading cause of cancer-related deaths worldwide.[1] The interaction between population growth, the change in aging structure, and the decline in incidence and mortality rates will lead to an increased burden of GC.[2] At present, chemotherapy is still the gold standard for patients with metastatic GC post-surgical resection. Commonly used chemotherapy drugs for GC treatment mainly include oxaliplatin and 5-fluorouracil (5-FU).[3] Poor or no response to chemotherapy is frequently observed in patients with GC due to the development of drug resistance, ultimately leading to clinical treatment failure and patient death.[4] Adenosine triphosphate-based tumor chemosensitivity assay can predict the clinical outcomes of taxane-based chemotherapy.[5] The underlying mechanisms of GC drug resistance are very complex and still not fully understood. Therefore, understanding the mechanisms of resistance to these drugs is needed to develop new approaches in individual diagnostic and therapeutic strategies for patients with GC.[6,7]

Long non-coding RNAs (lncRNAs) are a large class of endogenous ncRNAs over 200 nucleotides in length. They regulate the timing and extent of gene expression through multiple mechanisms, including signals, decoys, scaffolds, guides, and SINEUP, and get involved in proliferation, apoptosis, invasion, and metastasis.[8–12] In fact, dysregulation and aberrant expression of lncRNAs have been observed in GC samples and cell lines.[13,14] Growing evidence suggests that lncRNAs are involved in nearly all aspects of GC progression, including drug resistance.[15–17]

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DNA damage checkpoints function primarily at G1-S and G2-M, which regulate whether cells continue mitosis or undergo DNA repair or apoptosis.[18–20] Ataxia-telangiectasia mutated and Rad3 related (ATR) is one of the central regulators that controls the cellular response to DNA damage.[21,22] During the G2 phase, activation of the ATR/CHK1 pathway prevents mitosis in cells with damaged DNA.[23,24] Homologous recombination (HR) is the main pathway involved in DNA repair after antitumor drug treatment.[25] At the S-G2 stage, ATR and CHK1 control the G2/M checkpoint and enhance the HR pathway based on templates of intact sister chromatids.[26] The ATR inhibitor AZD6738 is currently used as a monotherapy and in combination with chemotherapy and radiotherapy in clinical trials with good prognoses, and it may be applied as a valuable and powerful anticancer agent in the near future.[27] The CHK1 inhibitor LY2606368 impairs HR-mediated DNA damage repair, thereby enhancing the antitumor efficacy of polyadenosine diphosphate-ribose polymerase (PARP) inhibitors.[28]

In this study, the expression and functional role of RP11-789C1.1 in GC were evaluated, and the mechanism by which RP11-789C1.1 sensitizes the anticancer effect of oxaliplatin was characterized. Furthermore, it was observed RP11-789C1.1 induced GC proliferation and apoptosis by regulating the ATR/CHK1 pathway.

Methods

Patients and specimens

Twenty fresh-frozen specimens were collected from patients with GC who underwent surgery in the First Affiliated Hospital of Sun Yat-sen University from 2019 to 2022. All patients were histologically diagnosed with GC. Gender, age, Tumor Node Metastasis (TNM) stage, and other data were collected and analyzed. Briefly, T describes the size of the original (primary) tumor and whether it has invaded nearby tissue, N denotes nearby (regional) lymph nodes that are involved, and M designates distant metastasis (spread of cancer from one part of the body to another). The Tumor Genome Atlas project (TCGA, https://www.cancer.gov/ccg/research/genome-sequencing/tcga) dataset of GC (STAD, gastric adenocarcinoma) was also downloaded. This study was approved by the Ethical Review Board of the First Affiliated Hospital of Sun Yat-sen University (No.[2021]287) and all patients gave written informed consent.

Cell lines and cell culture

Human GC cell lines (AGS, MKN-45, BGC-823, KATOIII, HGC-27, and SGC-7901) and normal gastric epithelial cells (GES-1) were obtained from Procell Life Science & Technology Co., Ltd., Wuhan, China and Zhong Qiao Xin Zhou Biotechnology Co., Ltd., Shanghai, China. All cell lines were verified by mycoplasma detection and short tandem repeat (STR) profiling. Cells were cultured in RMI-1640 supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, USA) and Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, USA) at 37°C in 5% CO2 atmosphere. To evaluate the effects of RP11-789C1.1 in ATR-CHK1 signaling pathway, the cells were treated with concentrations of AZD6738 (1 μmol/L , S7693, Sellleck, Texas, USA) for 12 hours.

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

Total RNA was extracted using the Trizol method, and complementary DNA (cDNA) synthesis was performed using PrimeScript RT Master Mix (Takara, Osaka, Japan) according to the manufacturer's instructions. qRT-PCR analysis was implemented using a fluorescence qRT-PCR instrument (Roche, Basel, Switzerland). In this study, all samples were taken in triplicate. Relative expression was determined using the $2^{-\Delta\Delta CT}$ method and normalized to the mean value of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers used in this study were detailed in the Supplementary Table 1, http://links.lww. com/CM9/B748.

Cell survival assay

GC cells (1×10^3 cells/well) were seeded in 96-well plates. Cell proliferation rate was determined by Cell Counting Kit-8 (CCK8). Ten microliters of CCK8 solution were added to each well. After incubation at 37°C for 2 h, the absorbance was examined using a microplate reader at 450 nm. The cell proliferation rate was calculated as (average optical density [OD] of treated wells) – OD of blank wells/(OD of control wells – OD of blank wells) × 100. Five technical replicates were prepared for each sample in three independent experiments.

Clonogenic assay

Cells (1×10^3 cells/well) overexpressing RP11-789C1.1 were seeded into 6-well plates, and the control group cells were incubated for 10 days. The cell clones were evaluated by ImageJ (V1.8.0.112, National Institutes of Health, Bethesda, USA). The experiment was carried out three times.

Western blot analysis

Radio immuno precipitation assay (RIPA) buffer contains phosphatase inhibitors and protease inhibitors for cell lysis. Equivalent amounts of protein were separated by 6–12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein bands were detected with enhanced chemiluminescence (ECL) detection reagent. The results were analyzed using ImageJ. The experiment was performed three times. Antibodies against CHK1 (1:1000, 2360, Cell Signaling Technology, Massachusetts, USA), P-CHK1S317 (1: 1000, 12302S, Cell Signaling Technology), and ATR (1: 1000, 19787-1-AP, Proteintech) were purchased from

Proteintech (Wuhan, China), and GAPDH was purchased from Cell Signaling Technology.

RNA-binding protein immunoprecipitation (RIP) assay

RIP assays were executed according to the instructions of Magna RIP Kit (Millipore, Burlington, MA, USA). In short, the cells were lysed with RIP lysis buffer and incubated with ATR or rabbit immunoglobulin (Ig) G-specific antibodies. Co-precipitated RNA was detected by qRT-PCR with specific primers. Rabbit IgG was used as a reference.

Pull-down assay with biotinylated RP11-789C1.1 probe

RNA pull-down assay was achieved using a Pierce™ Magnetic RNA-Protein Pull-Down Kit (Thermo Scientific, Waltham, USA). Briefly, AGS or HGC-27 cells (1×10^7) were harvested and lysed in immunoprecipitation lysis buffer on ice for 30 min. The biotin-labeled RP11-789C1.1 probe and the control probe (GenePharma, Shanghai, China) were incubated at 25°C for 30 min to generate probe-coated beads. The cell lysate was incubated with RP11-789C1.1 probe or control probe followed by washing and elution to extract the protein bound to RP11-789C1.1. The protein pulled down by the probe was used for Western blot analysis and mass spectrometry. We employed The Fast Silver Stain Kit for staining (P0017S, Beyotime, Shanghai, China), and specific experimental steps were carried out following the protocol provided by the kit.

Apoptosis and cell cycle analysis

To investigate cell viability, cells were plated in 96-well plates at a density of 3000 cells/well and treated with oxaliplatin (4 μg/mL, S1224, Selleck, Shanghai, China) for 24 h. The cells were collected by trypsin digestion and were washed twice with phosphate buffered saline (PBS) at 4°C to observe apoptosis. The proportion of apoptotic cells was detected by flow cytometry. These experiments were performed using three biological replicates. Analysis was achieved with FlowJo (Becton, Dickinson and Company, Franklin Lakes, USA).

Cell transfection and RNA interference

The RP11-789C1.1 overexpression plasmid was used to package HEK 293 T cells (obtained from ATCC, Manassas, USA) with Lentit-PAC HIV Expression Packaging Kit (GeneCopoeia, Guangzhou, China) and was used to infect GC cell lines. Stably transfected cells were constructed using 4 μg/mL puromycin [Supplementary Table 2 and Material, http://links.lww.com/CM9/B748].

In vivo experiments

The Animal Care and Utilization Committee of the First Affiliated Hospital of Sun Yat-sen University approved all animal experiments in this study (No. [2021]046). Control and RP11-789C1.1 overexpressed (OV) cells were resuspended in PBS to obtain a concentration of 1×10^7 cells/mL, and then 150 μL of this solution was injected subcutaneously into the right abdomen of each mouse.

Gene set enrichment analysis (GSEA)

Based on the TCGA dataset of GC (STAD), GSEA was performed using GSEA (version 3.0, Broad Institute, Massachusetts Institute of Technology, Cambridge, USA) to identify related biological processes. The null distribution of enrichment score (ES) was generated using 1000 gene permutations. Then, the normalized enrichment score (NES) was calculated for each enrichment path. False detection rate (FDR) <5% and *P*-value <0.05 were considered statistically significant.

Statistical analyses

SPSS (version 22.0, International Business Machines Corporation, Armonk, USA) and GraphPad Prism 8 version (Dotmatics, Boston, USA) were used for statistical analysis. Quantitative data were expressed as mean ± standard deviation. Statistical significance was determined by two-tailed Student's *t*-test or one-way ANOVA. Each experiment was repeated at least three times. *P*-value <0.05 was considered statistically significant.

Results

Expression of RP11-789C1.1 was decreased in GC tissues and cell lines

In our previous study, we used microarray data to screen for lncRNAs that were differentially expressed in GC, including RP11-789C1.1.[29] To clarify the important role of RP11-789C1.1 in the occurrence and development of GC, we first used tissue samples to detect the expression level of RP11-789C1.1. The results indicated that the expression level of RP11-789C1.1 in GC tissues was significantly decreased as compared with that in paired gastric tissues [Figure 1A]. Next, we evaluated the differential expression of RP11-789C1.1 in multiple GC cell lines and normal gastric epithelial cell lines. Similar to the tissues,

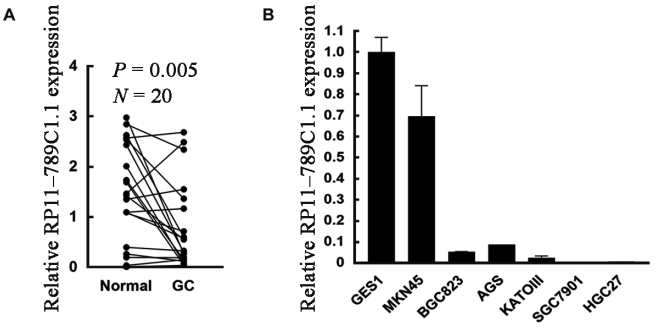

Figure 1: RP11-789C1.1 was downregulated in GC. (A) Expression levels of RP11-789C1.1 in GC and adjacent tissues were determined by qRT-PCR. (B) Expression levels of RP11-789C1.1 in GC cell lines and normal gastric epithelial cells line were revealed by qRT-PCR. GC: Gastric cancer; qRT-PCR: Quantitative real-time polymerase chain reaction analysis.

the expression of RP11-789C1.1 was significantly decreased in GC cell lines [Figure 1B].

RP11-789C1.1 inhibited GC cell proliferation and promoted apoptosis

We transfected RP11-789C1.1 overexpression plasmids in AGS and HGC27 GC cell lines and verified the overexpression efficiency [Figure 2A]. CCK8 and colony formation experiments demonstrated that the growth and proliferation rates of AGS and HGC27 cells were significantly decreased after overexpression of RP11-789C1.1 [Figures 2B, C]. Flow cytometry apoptosis analysis indicated that apoptosis of AGS and HGC27 cells was significantly enhanced after overexpression of RP11-789C1.1 [Figure 2D]. In addition, subcutaneous xenograft experiments in nude mice confirmed that RP11-789C1.1 could inhibit the growth of GC cells *in vivo* [Figure 2E].

RP11-789C1.1 interacted with ATR

To further explore the specific mechanism of RP11-789C1.1 inhibitive role in the biological behavior of GC cells, we conducted a pull-down experiment [Figure 3A]. Compared with the control group, a total of 79 proteins were pulled down in the RP11-789C1.1 probe group [Supplementary Table 3, http://links.lww.com/CM9/B748]. Adopting bioinformatics analysis, we found that ATR expression was significantly elevated in GC [Figures 3B, C]. Therefore, we selected the ATR protein as the potential target of RP11-789C1.1 and implemented pull-down combined RIP experiments to prove the interaction between RP11-789C1.1 and ATR [Supplementary Figures 1A, B, http://links.lww.com/CM9/B748]. Further GESA analysis according to the expression of ATR disclosed that ATR could be involved in various signaling pathways [Figure 3D].

RP11-789C1.1 downregulated checkpoint kinase 1 (CHK1) phosphorylation via ATR

ATR functioned by regulating the phosphorylation of CHK1, and we hypothesized that RP11-789C1.1 could regulate the downstream target molecule CHK1 of ATR. CHK1 was up-regulated in GC according to the TCGA database [Figure 4A]. Western blot analysis results revealed that the phosphorylation of CHK1 was significantly decreased after overexpression of RP11-789C1.1, suggesting that RP11-789C1.1 exerted a tumor suppressor function by regulating the phosphorylation of CHK1 [Figure 4B]. Considering the high expression of RP11-789C1.1 in the MKN45 cell line, we further used the MKN45 cell line as a positive control to detect the protein expression levels of ATR, CHK1, and p-CHK1 to clarify the regulatory effect of RP11-789C1.1 on CHK1 in GC cell lines. The results illustrated that both overexpression of RP11-789C1.1 and high expression of RP11-789C1.1 could down-regulate the expression of p-CHK1 [Supplementary Figure 1C, http://links.lww.com/CM9/B748]. We then knocked down RP11-789C1.1 in the MKN45 cell line and found that the level of p-CHK1 was higher than that in the control group. In addition, it was determined that the combination of si-RP11-789C1.1 and ATR inhibitor AZD6738 led to a significant decrease in the expression of p-CHK1 [Supplementary Figures 2A, B, http://links.lww.com/CM9/B748], which verified that AZD6738 could abolish the increase in the expression of p-CHK1 caused by si-RP11-789C1.1, thus confirming that RP11-789C1.1 regulated the expression of p-CHK1 through ATR.

RP11-789C1.1 mediated oxaliplatin sensitivity through ATR/CHK1

We treated GC cells with oxaliplatin and observed that as compared with the control group, the GC cells in the overexpression RP11-789C1.1 group showed inhibited growth and proliferation, promoted apoptosis [Figures 5A, B], and significantly downregulated phosphorylation of CHK1 [Figure 5C] after treatment with oxaliplatin. Therefore, RP11-789C1.1 mediated oxaliplatin sensitivity in GC by regulating CHK1 phosphorylation [Figure 6].

Discussion

Most patients with GC are diagnosed at an advanced stage of the disease and generally have a poor prognosis. The current clinical research results of targeted therapy and immunotherapy for GC are largely unsatisfactory due to the high heterogeneity of GC and the rapid drug resistance to targeted drugs.[30] Chemotherapy prolongs the overall survival of patients with advanced GC; however, the resistance of GC cells to drugs leads to poor prognoses. The complex mechanisms involved in drug resistance in GC include inactivation of apoptosis signaling pathways and DNA damage repair capabilities, loss of cell cycle checkpoint control, increased drug efflux and decreased uptake, and phenotypic transitions such as epithelial-mesenchymal transition (EMT).[31–33] However, the detailed mechanisms of drug resistance in GC remain inconclusive.

LncRNAs are involved in regulating cancer chemoresistance.[34] LncRNAs regulate drug resistance by acting as competing endogenous RNAs (ceRNAs) or directly binding to messenger RNAs (mRNAs) or proteins and regulating their expression and/or function. In GC, various aberrantly expressed lncRNAs serve as diagnostic and prognostic markers[35,36] and have been identified as promoting tumor progression, radioresistance, chemoresistance, and targeted therapy sensitivity.[37–42]

In our study, RP11-789C1.1 inhibited the growth and proliferation of GC cells and promoted the apoptosis of GC cells *in vitro* and *in vivo*, indicating that RP11-789C1.1 could be used as a therapeutic target for GC. In addition, RP11-789C1.1 regulated the sensitivity of GC cells to oxaliplatin, indicating that RP11-789C1.1 played an important role in GC chemotherapy resistance.

ATR promotes cell survival through various ways including promoting DNA repair (including HR), cell

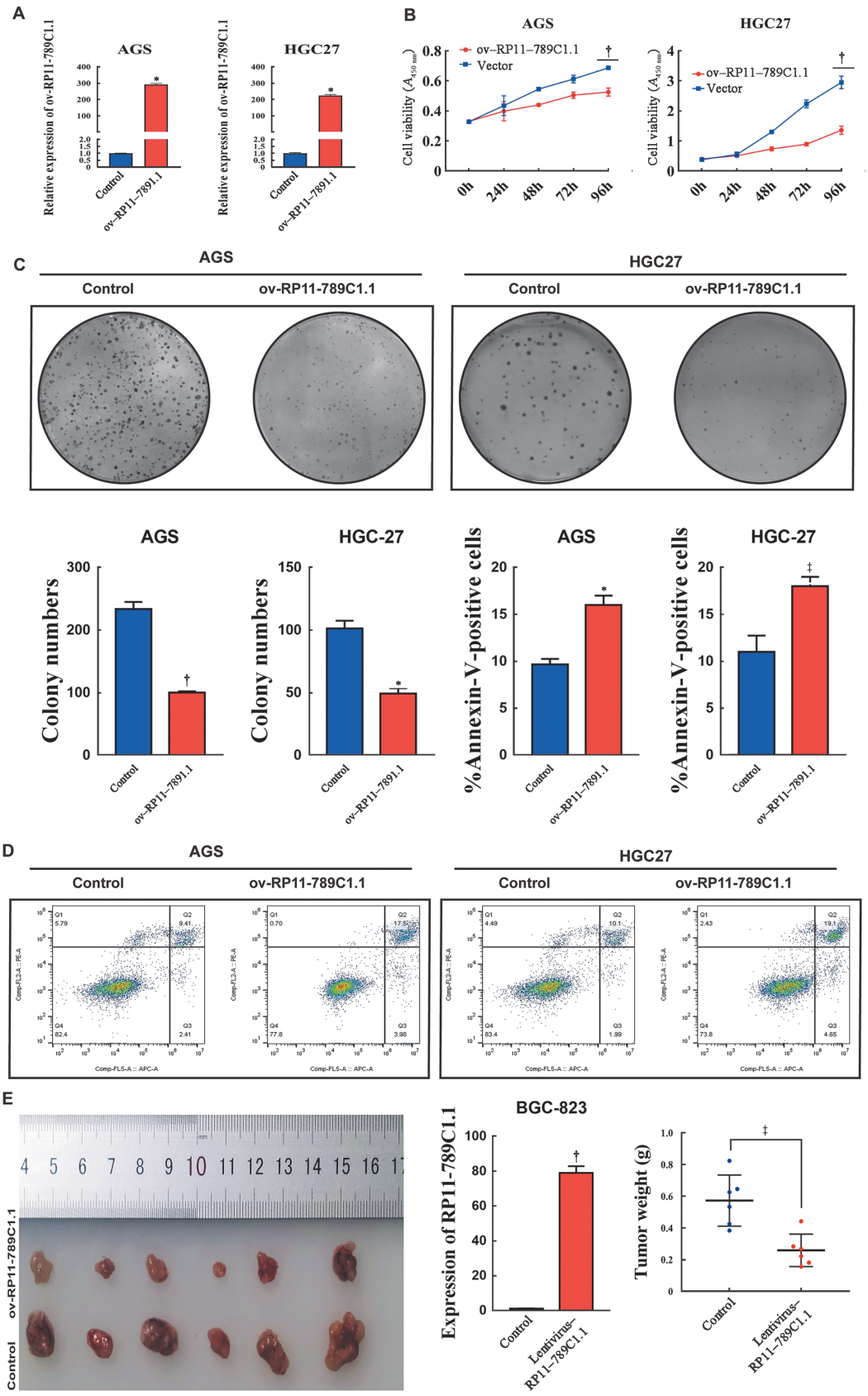

Figure 2: RP11-789C1.1 inhibited the biological behaviors of GC. (A) The level of RP11-789C1.1 in GC cells transfected with the indicated plasmid was determined by qRT-PCR. (B–C) The effect of RP11-789C1.1 overexpression on the proliferation potential of gastric cancer cells was examined by CCK8 and colony assays. (D) The effect of RP11-789C1.1 overexpression on the apoptosis of GC cells was examined by flow cytometry. (E) The effect of overexpression of RP11-789C1.1 on the growth of GC cells *in vivo* was confirmed by subcutaneous tumorigenesis experiments. $^{*}P<0.001$, $^{\dagger}P<0.0001$, and $^{\ddagger}P<0.01$. CCK8: Cell Counting Kit-8; GC: Gastric cancer; qRT-PCR: Quantitative real-time polymerase chain reaction analysis.

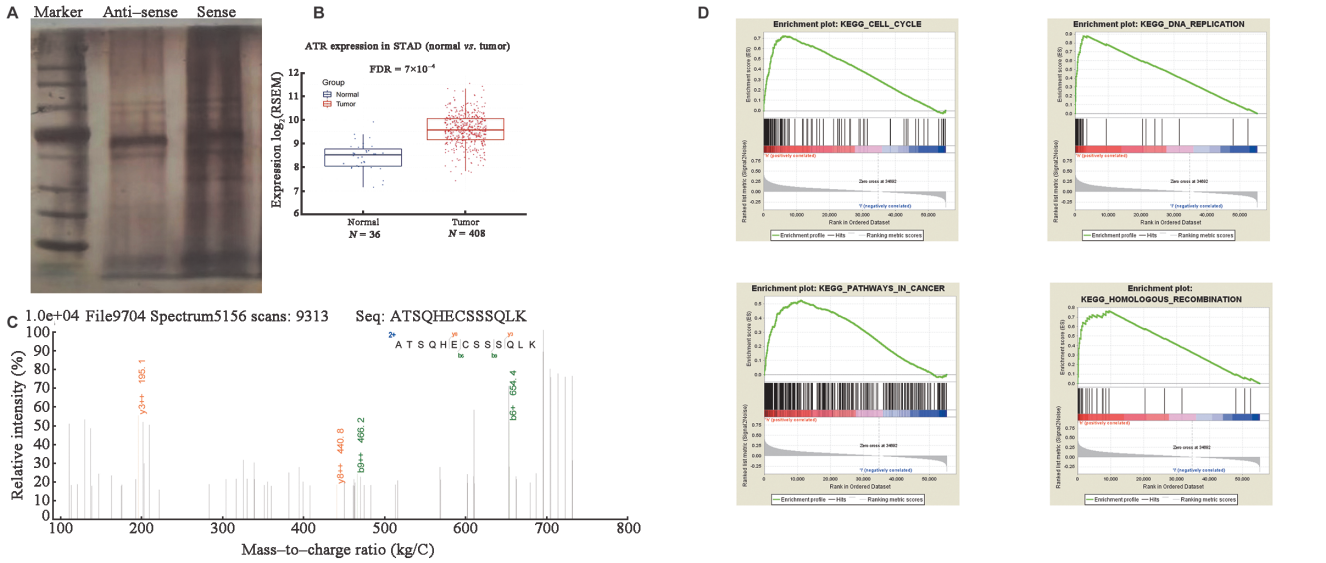

Figure 3: RP11-789C1.1 interacted with ATR. (A) The silver staining of RP11-789C1.1 binding protein by RNA pull-down technique was performed. (B) Identification of the upregulated expression of ATR in GC through the TCGA database (N_{Normal} = 36; N_{Tumor} = 408). (C) Mass spectrogram for identification of ATR protein binding to RP11-789C1.1. (D) GSEA analysis by ATR expression level revealed the enrichment of cancer, cell cycle, HR, and DNA replication-related pathways through the TCGA database. ATR: Ataxia-telangiectasia mutated and Rad3 related; FDR: False discovery rate; GC: Gastric cancer; GSEA: Gene set enrichment analysis; HR: Homologous recombination; TCGA: Tumor Genome Atlas Project.

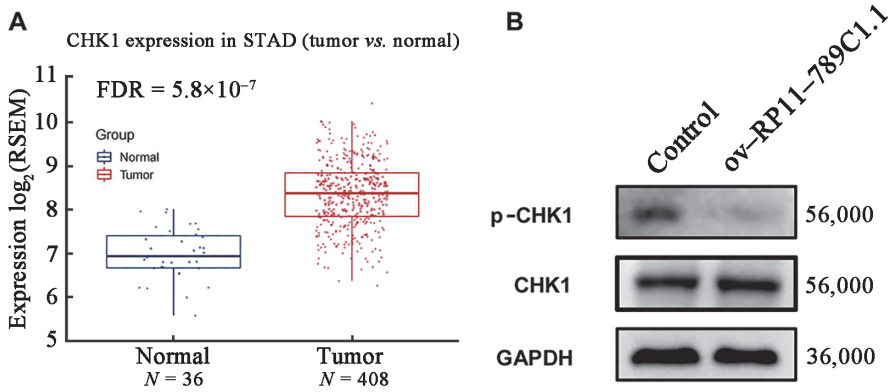

Figure 4: RP11-789C1.1 downregulated CHK1 phosphorylation via ATR. (A) Identification of the up-regulated expression of CHK1 in GC through the TCGA database (N_{Normal} = 36; N_{Tumor} = 408). (B) Effect of overexpression of RP11-789C1.1 on CHK1 phosphorylation. FDR: False discovery rate; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; GC: Gastric cancer; RSEM: RNA-Seq by expectation maximization; STAD: Stomach adenocarcinoma; TCGA: Tumor genome atlas project.

cycle arrest, and stabilizing stalled replication forks. ATR plays a vital part in many tumor cells compared with normal cells.[43–45] The combination of ATR inhibitors and genotoxic-inducing chemotherapeutics including cisplatin, gemcitabine, topoisomerase I poisons, etoposide, and oxaliplatin.[46–50] The combination of oxaliplatin and an ATR inhibitor (AZD6738) enhanced antitumor efficacy *in vivo* and produced a potent synergistic effect.[51]

Our study demonstrated that RP11-789C1.1 could interact with ATR to reduce CHK1 phosphorylation. Further research established that RP11-789C1.1 could promote the sensitivity of GC cell lines to oxaliplatin by regulating the ATR/CHK1 signaling pathway. Therefore, RP11-789C1.1 could be used as part of a combined therapeutic target for the treatment of GC.

Although the research demonstrated the potential of RP11-789C1.1 as a key molecule enhancing the anti-tumor effects of oxaliplatin in GC cells, it's important to acknowledge that the mechanism might be influenced by various patient-specific factors, tumor heterogeneity, and other molecular interactions. Additional in-depth studies, including investigations into the interplay between RP11-789C1.1 and other relevant biomolecules, are needed.

In conclusion, RP11-789C1.1 was lowly expressed in GC tissues and cells and was closely related to apoptosis and DNA damage repair pathways. Overexpression of RP11-789C1.1 significantly induced DNA damage and promoted apoptosis by inhibiting ATR/CHK1 pathway activation. Importantly, RP11-789C1.1 was a key molecule that

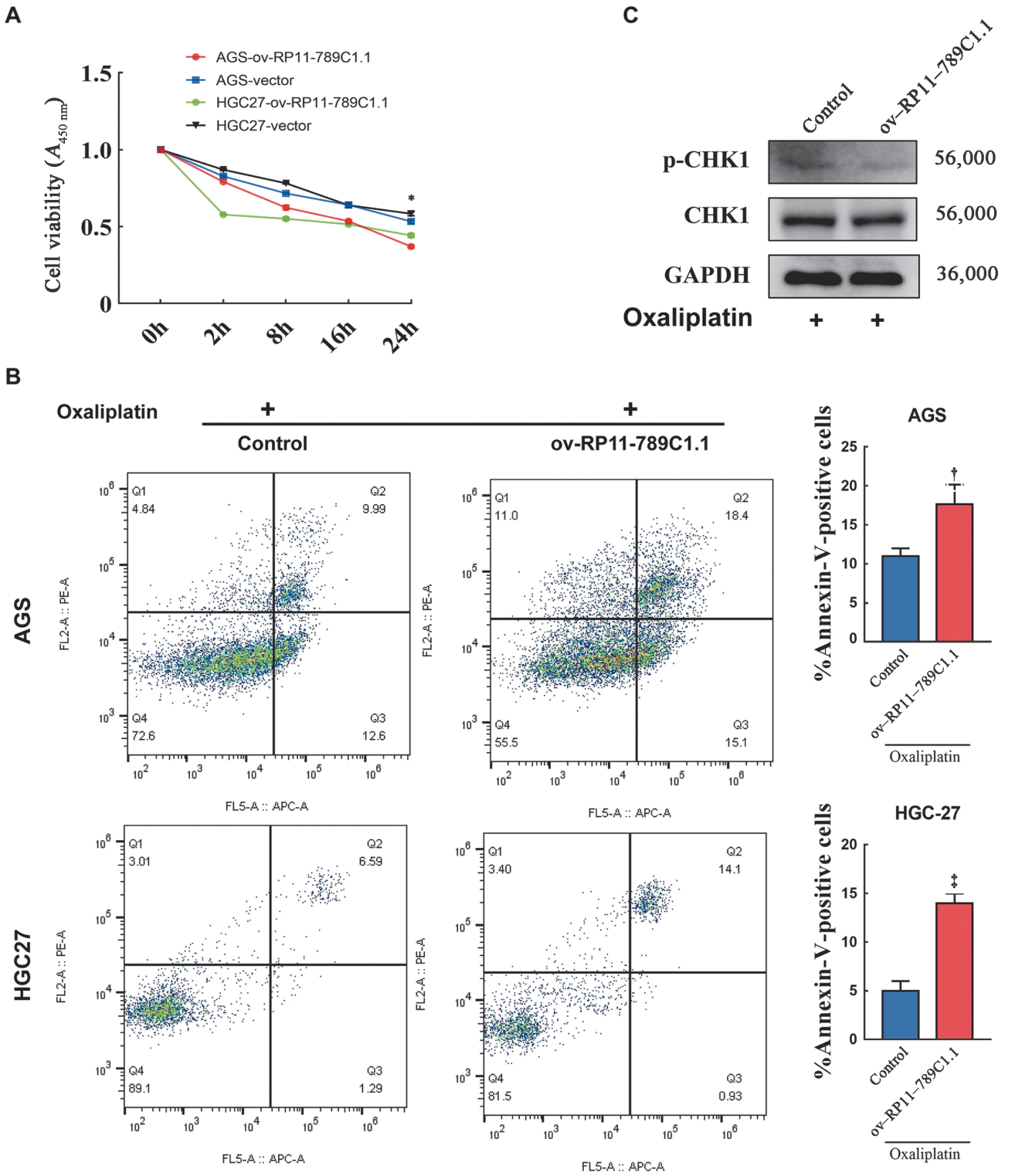

Figure 5: RP11-789C1.1 mediated oxaliplatin sensitivity. (A–B) The effect of overexpression of RP11-789C1.1 on oxaliplatin sensitivity was examined by cell viability test and flow cytometry. (C) The effect of overexpression of RP11-789C1.1 on CHK1 phosphorylation after treatment with oxaliplatin. $^{*}P$ <0.0001, $^{\dagger}P$ <0.05, and $^{\ddagger}P$ <0.001. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

enhanced the antitumor effect of oxaliplatin on GC cells. Therefore, RP11-789C1.1 combined with oxaliplatin may be a promising therapeutic strategy for the treatment of GC.

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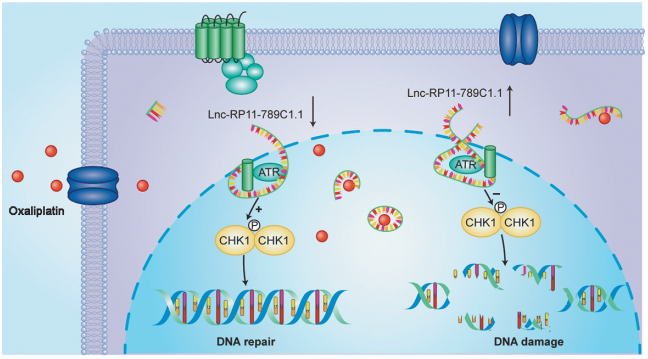

Figure 6: Schematic map of the mechanism RP11-789C1.1 in GC. GC: Gastric cancer. ATR: Ataxia-telangiectasia mutated and Rad3 related; CHK1: Checkpoint kinase 1.

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Conflicts of interest

None.

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