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ORIGINAL ARTICLE

Mechanism of RBBP8-mediated homologous recombination repair in gastric cancer synthetic lethal

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Abstract

Background: It is of great clinical significance to further explore new strategies and potential combined therapeutic targets for gastric cancer. This study aimed to investigate the synthetic lethal effect of RBBP8 molecular intervention combined with a poly ADP ribose polymerase (PARP) inhibitor in non-BRCA mutant gastric cancer and clarify the mechanism by which RBBP8 regulates homologous recombination repair.

Methods: The role of RBBP8 in DNA damage repair was observed using bioinformatic analysis, western blot analysis, and immunofluorescence. The synthetic lethal effect was verified using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS)and flow cytometry apoptosis experiments.

Results: Among the patients with gastric cancer treated with chemotherapy, the prognosis of patients with high RBBP8 expression levels was worse (homologous recombination [HR] = 1.54, $p = 0.028$). RBBP8 knockdown induced DNA damage and had a synergistic effect with PARP inhibitor treatment on cell viability inhibition and cell apoptosis in AGS (generic code for human gastric adenocarcinoma cells) ($t = 11.154$, $p < 0.001$) and N87 ($t = 6.362$, $p < 0.001$) cells. RBBP8 knockdown inhibited RAD51 activation and DNA terminal excision in homologous recombination repair.

Conclusion: RBBP8 is involved in homologous recombination repair, and molecular intervention into RBBP8 could achieve a synthetic lethal effect with PARP inhibitor treatment in gastric cancer cells.

KEYWORDS

gastric cancer, homologous recombination repair, molecular mechanism, RBBP8, synthetic lethality

Highlights

- RBBP8 protein expression and phosphorylation modification are cell cycle-dependent, which is similar to homologous recombination (HR) repair.
- We proved that RBBP8 participates in the key steps of HR repair and verified the synthetic lethal effect of RBBP8 molecular intervention and PARP inhibitor treatment in gastric cancer.
- The research would contribute to broadening the application of PARP inhibitor treatment in non-BRCA mutant gastric cancer and solve its clinical drug resistance problem.

Yang Yu and Shuxia Wang contributed equally to this study.

1 | INTRODUCTION

According to the 2020 Global Cancer Report, the annual incidence of gastric cancer ranks fifth in the world, while the number of gastric cancer-related deaths ranks fourth.[1] The gastric cancer incidence rate in China is nearly three times the global average, accounting for nearly half of new gastric cancer cases worldwide.[2] In recent years, with the improvement of national health awareness and progress in digestive endoscopy diagnosis and treatment technology, an increasing number of gastric cancer cases have been successfully diagnosed and treated early. However, due to the unbalanced development of China's medical system and endoscopic physicians' diagnosis abilities, the overall diagnosis rate of early gastric cancer in China is only 10%–15%.[3] The advent of a series of molecular targeted drugs (such as trastuzumab) has improved gastric cancer treatment, but the therapeutic effect of a single targeted drug is limited and only effective in a small number of patients (6.7%–22.9%).[4–6] Therefore, it is of great clinical significance to further explore new strategies and potential combined therapeutic targets for gastric cancer.

RBBP8 is a nuclear protein that is commonly expressed in eukaryotic cells.[7] Previous studies have shown that RBBP8 is involved in the formation of the DNA double-strand break repair complex.[8] The repair mode of DNA double-strand damage is specific to the cell cycle. Homologous recombination (HR) repair with high fidelity mainly occurs in the late S and G2 phases.[9,10] However, the specific mechanism by which HR repair achieves high cycle selectivity regulation is unclear. Our previous research showed that RBBP8 can achieve deacetylation of the P21 promoter and inhibit its transcription through protein interaction and chromatin modification mechanisms to promote the G1/S phase transition; RBBP8 expression is cell cycle-dependent, similar to HR repair.[11] Therefore, we hypothesize that RBBP8 is the key protein in the precise regulation of HR repair. This study will further investigate the mechanism of RBBP8 participation in HR repair.

Poly ADP ribose polymerase (PARP) inhibitors can interfere with the BER repair pathway in DNA single-strand damage to kill tumor cells; they are currently the most widely studied single-strand damage repair pathway inhibitors.[12,13] When the repair function of DNA single-strand damage is inhibited, it gradually develops into double-strand damage. If tumor cells simultaneously have defects in double-strand damage repair function, the "synthetic lethal" effect could kill the tumor. A previous study found that tumors with BRCA mutations (with natural defects of HR repair) are highly sensitive to PARP inhibitors.[14] In addition, the exploration of new targets for HR repair defects based on the synthetic lethal principle has also become a research hotspot in tumor therapy. This study aimed to verify the synthetic lethal effect of RBBP8 intervention and PARP inhibitor treatment in gastric cancer, clarify the specific mechanism of RBBP8 in HR repair pathway regulation, and provide new strategies and candidate targets for the application of RARP inhibitors in patients with non-BRCA mutation gastric cancer.

2 | MATERIALS AND METHODS

2.1 | Bioinformatic analysis

We searched the GEO database for relevant survival information about patients with gastric cancer after chemotherapy, analyzed the survival of 118 eligible patients in the GSE14210 data set, and drew Kaplan–Meier survival curves.

2.2 | Western blot analysis

The gastric cancer cell line AGS (generic code for human gastric adenocarcinoma cells) (purchased from Shanghai Institute of Cell Biology, Shanghai, China) was infected with sh-RBBP8 and blank control, and the stable infected cell line was screened. Western blot analysis was performed to verify the knockdown efficiency of the RBBP8 protein. The protein samples were prepared at a ratio of 40 μg: 20 μL, and electrophoresis was performed (concentrated gel, 80 V for 30 min; separated gel, 100 V for 60 min); transmembrane was performed at 390 V for 70 min. The membrane was blocked with 5% skimmed milk for 1–2 h. The first antibody was incubated overnight in a refrigerator at 4°C and the second antibody at room temperature. Western blot analysis was carried out in gastric cancer cell lines AGS and N87 (purchased from Procell Life Science and Technology Co, Ltd.) to observe the DNA damage marker γ-H2AX levels (Abcam #ab229914) after RBBP8 protein knockdown. After cell cycle synchronization, the protein extracted at each time point was subjected to western blot analysis to observe RBBP8 protein phosphorylation (Abcam #ab254067) during different phases of the cell cycle.

2.3 | Cell cycle synchronization

The G0 phase of the cell cycle was synchronized using the contact inhibition method and serum-free medium treatment. AGS cells were cultured in normal medium until the "over-fusion" state, then cultured in F12K/DMEM medium without fetal bovine serum for 24 h. After digestion, cells were collected as the 0-h group, and the remaining cells were reseeded into a 6-well cell culture plate with a relatively sparse density. At 6, 12, 16, 20, 24, 30, and 36 h, AGS cells were digested and

collected separately. For G1 phase synchronization, the TdR double-blocking method was used. For G2 phase synchronization, nocodazole (1 μg/mL, Sigma #31430-18-9) was used. Proteins were extracted from G1 and G2 phase cells, and RBBP8 phosphorylation was judged by the "dragging degree" of the band.

2.4 | Cell phenotype test

First, to assess 3–(4,5–dimethylthiazol–2–yl)–5–(3–carboxymethoxyphenyl)–2–(4–sulfophenyl)–2H–tetrazolium, inner salt (MTS) cell viability, six concentration gradients were designed for PARP inhibitor treatment BMN673: 0.01, 0.1, 0.5, 1.0, 5.0, and 10.0 μmol/L. Each group had four multiple holes and 3000 cells. The optical density (OD) value detected 6 h after seeding was taken as the standard value. After 72 h of treatment, MTS reagent was added to measure the OD of each well.

Second, the cell apoptosis test was carried out. After 72 h of treatment, cells were collected for staining, including the complete blank, Annexin V-FITC single-positive (BD Pharmingen #9197303), 7-AAD single-positive (BD Pharmingen #9197303), and double-positive groups. Annexin V-FITC-7-AAD double-positive staining was observed using FACS Verse (Becton-Dickinson). The Annexin V single-stained and double-stained cells were counted as the apoptotic percentage.

2.5 | Immunofluorescence testing

The experimental groups included the negative control, PARP inhibitor, Sh-RBBP8 knockdown, Sh-RBBP8 + PARP-inhibitor, and pCMV-RBBP8 overexpression amelioration groups. The activation of RAD51 (Alexa Fluor 488 goat anti-mouse) and γ-H2AX (Alexa Fluor 568 goat anti-rabbit) aggregation induced by PARP inhibitor treatment was observed and photographed using a confocal microscope (IX83, FLUOVIEW FV1200, Olympus). Cells were incubated with 5-bromodeoxyuridine (BrdU, Alexa Fluor 488 goat anti-mouse) and treated with 5 Gy radiation for 4 h to observe the effect of si-RBBP8 knockdown on single-stranded DNA formation.

2.6 | Statistical analysis

SPSS 19.0 (IBM Corp) and GraphPad Prism 5 (GraphPad Software) were used for statistical analysis and visualization. At least three independent experiments were performed in triplicate and statistical significance was set at $p < 0.05$.

3 | RESULTS

3.1 | RBBP8 is involved in cell cycle-specific DNA damage repair

Survival analysis of 118 patients in the GSE14210 data set with gastric cancer treated with chemotherapy showed that the prognosis of patients with high RBBP8 expression levels is worse than low group (HR = 1.54, $p = 0.028$, Figure 1A), suggesting that DNA damage repair activities related to RBBP8 might be involved in chemotherapy resistance. In the gastric cancer cell lines AGS and N87, after transient Si-RBBP8 transfection and stable Sh-RBBP8 lentivirus knockdown, γ-H2AX expression was significantly upregulated (Figure 1B). This suggests that the loss of RBBP8 protein expression caused a defect in DNA damage repair and promoted DNA damage. RBBP8 phosphorylation is highly cell-cycle dependent: it increases when the cell crosses the G1/S phase and maintains a high level during the S/G2 phase (Figure 1C). After TdR double blocking (G1 phase synchronization) and administration of 1 μg/mL nocodazole (G2 phase synchronization), the RBBP8 phosphorylation level in AGS cells was detected; the results show that RBBP8 phosphorylation modification almost exclusively took place during the S/G2 period (Figure 1D). This characteristic is highly consistent with the cycle selectivity of HR repair, which is only performed during the S/G2 phase in DNA double-strand breaks.

3.2 | RBBP8 participates in the key step of HR repair

Intranuclear RAD51 activation plays an important role in HR repair. We further explored the regulatory role of RBBP8 in the implementation of the HR repair pathway. PARP inhibitor treatment induced DNA damage in gastric cancer cell lines (H2AX fluorescence enhancement) and RAD51 activation in HR repair; however, Si-RBBP8 knockdown inhibited this intranuclear RAD51 aggregation, and exogenous RBBP8 plasmid overexpression ameliorated the RAD51 activation reaction (Figure 2A). The end excision of double-stranded broken DNA is the initial stage of HR repair, which can be detected by BrdU staining after the end excision. Under the condition of DNA damage induced by 5 Gy radiation, Si-RBBP8 knockdown inhibited the intranuclear staining of BrdU in AGS cells ($t = 7.943$, $p < 0.001$) and its intranuclear co-location with γ-H2AX (Figure 2B).

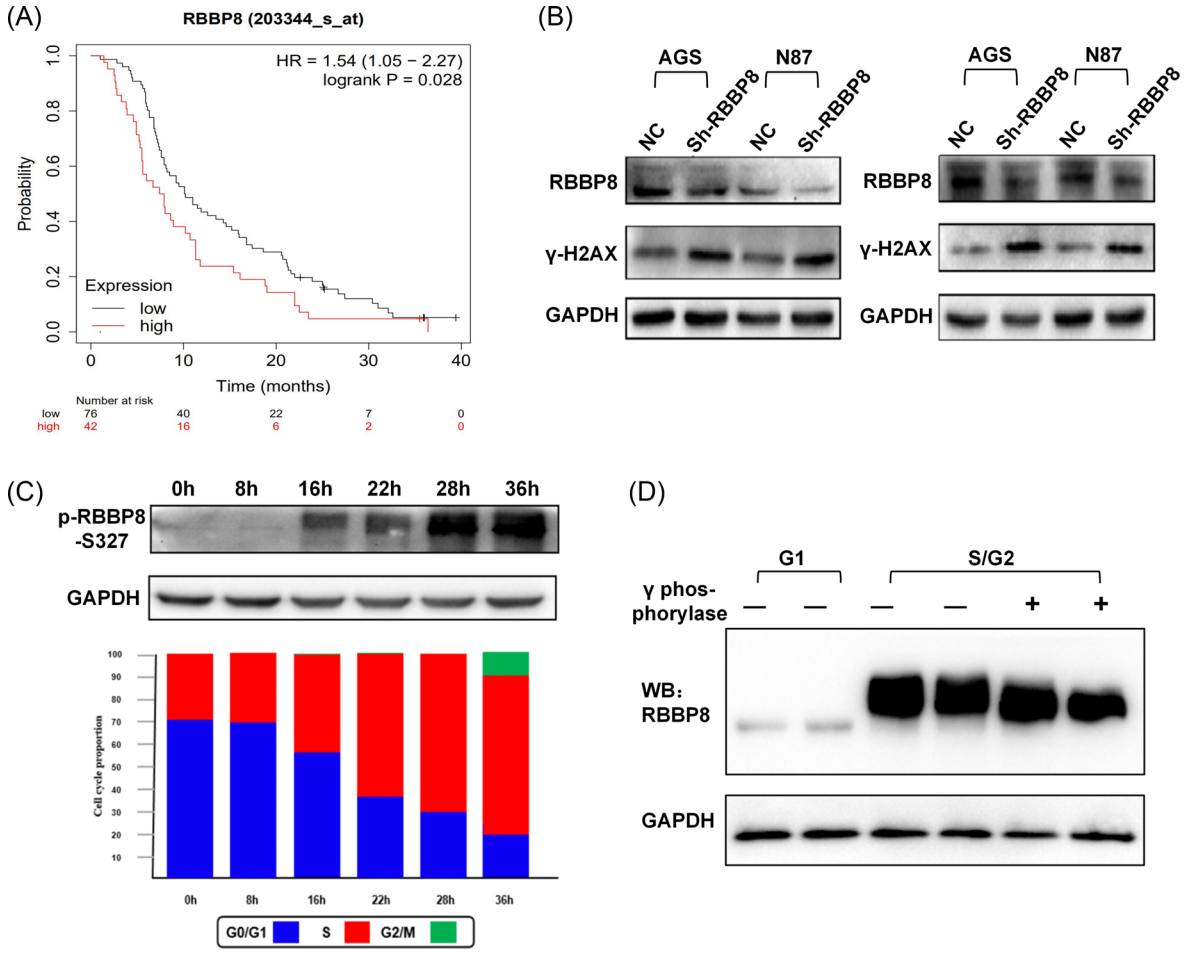

FIGURE 1 The prognosis was worse for patients with gastric cancer with high RBBP8 expression levels treated with chemotherapy (A). Decreased RBBP8 expression caused significant upregulation of γ-H2AX expression (B). RBBP8 phosphorylation modification mainly occurred in the S/G2 phase (C, D).

3.3 | RBBP8 knockdown and PARP inhibitor treatment have a synthetic lethal effect

To further verify the synthetic lethal potential of RBBP8 molecular intervention in gastric cancer, we carried out relevant cell phenotype experiments. MTS experiments showed that with increased PARP inhibitor (BMN673) concentration (0.01–10.00 μmol/L), the viability of gastric cancer cell lines AGS and N87 gradually decreased, and combination with RBBP8 knockdown further inhibited gastric cancer cell viability in AGS (Figure 3A) and N87 (Figure 3B) cells. This synergistic effect was further verified by flow cytometry apoptosis experiments, which showed that RBBP8 protein knockdown and PARP inhibitor treatment jointly promoted apoptosis in gastric cancer cells AGS ($t = 11.154$, $p < 0.001$, Figure 3C) and N87 ($t = 6.362$, $p < 0.001$, Figure 3D).

4 | DISCUSSION

DNA double-strand breaks (DSBs) caused by various pathological factors and ionizing radiation can lead to cell mutation, chromosome aberration, or even cell death. Eukaryotic cells can repair DNA damage through a variety of mechanisms.[15,16] The cell cycle stage is the key factor in determining how DNA double-strand breaks are repaired. RAD51 aggregation induced by DNA damage mainly occurs during the S/G2 phase.[17] During the G1 phase, DNA double-strand damage repair is carried out through nonhomologous or microhomology-mediated end joining.[18] These pathways usually involve DNA sequence loss at the break site. While in the late S phase and G2 phase, high-fidelity HR repair plays a leading role,[9,10] sister chromatids formed by DNA replication provide high-fidelity homologous templates for HR repair, which is extremely important to ensure genome stability throughout the repair process.

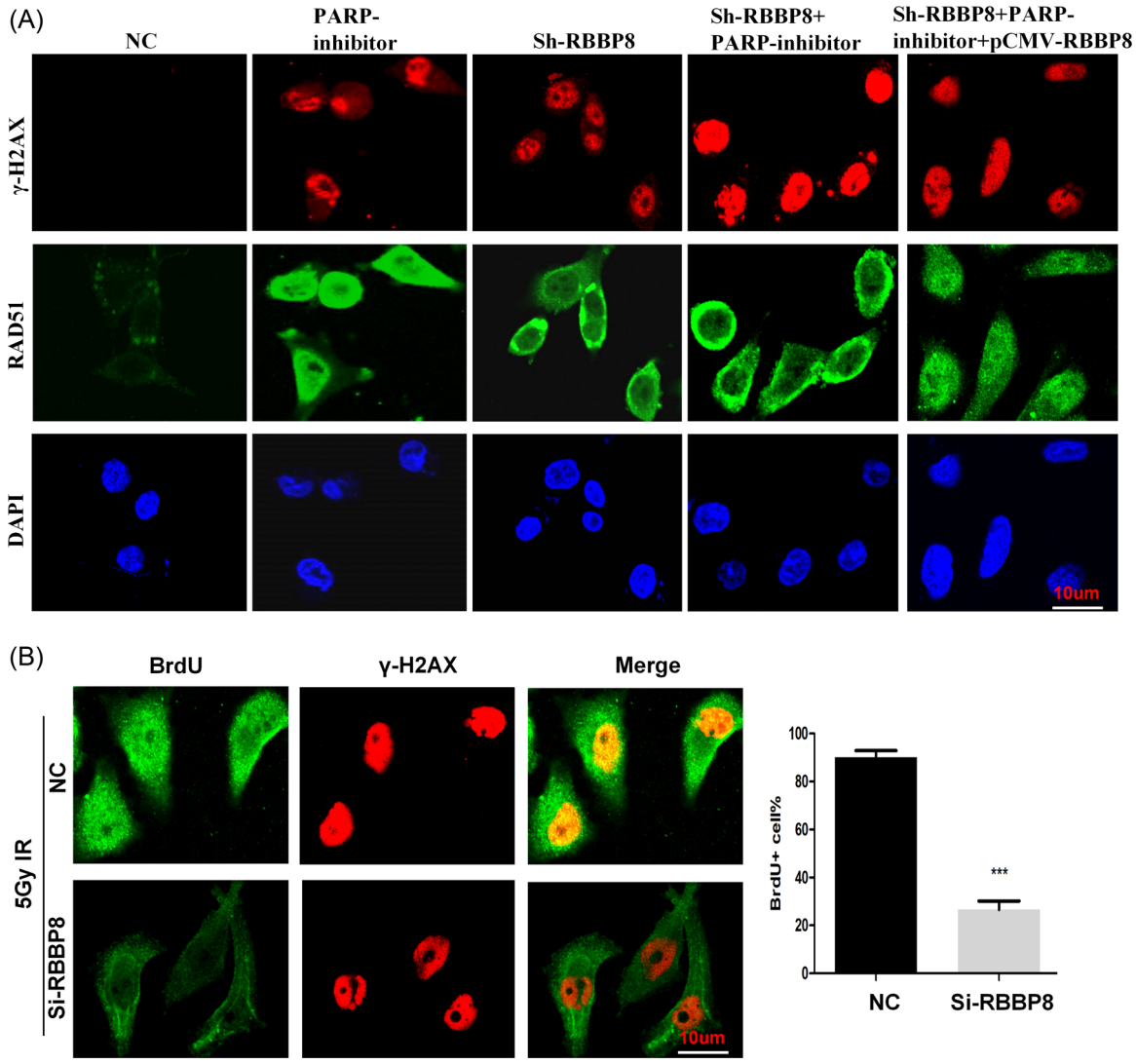

FIGURE 2 RBBP8 knockdown inhibited RAD51 activation induced by PARP inhibitors, and exogenous RBBP8 overexpression ameliorated this (A). Si-RBBP8 knockdown inhibited the intranuclear staining of BrdU and its intranuclear colocalization with γ-H2AX (B). Scale bars = 8 mm. ***p value < 0.001. PARP, poly ADP ribose polymerase.

Therefore, there should be a precise regulatory mechanism between the G1/S phase transition and HR repair execution.

Our research group has shown that RBBP8 can inhibit P21 transcription and activate CDK4/Cyclin d1 through chromatin modification to promote the G1/S phase transition of gastric cancer cells. Furthermore, RBBP8 protein expression is highly consistent with the cycle selectivity (S/G2 phase) of HR repair execution, suggesting that RBBP8 may play a key role in guiding DNA-damaged cells into the HR repair pathway. In this study, we first analyzed the survival of a gastric cancer cohort from the GEO database containing chemotherapy information from bioinformatics analysis. We found that the prognosis of the group with high RBBP8 expression levels was worse (Figure 1A), which suggests that RBBP8-related DNA damage repair activity might be involved in the chemotherapy resistance of patients with gastric cancer. In the western blot experiment, when the RBBP8 protein level was stably knocked down in two gastric cancer cell lines, AGS and N87, the γ-H2AX level, a DNA damage marker, increased significantly (Figure 1B). This shows that RBBP8 protein deletion affected the DNA damage repair process in gastric cancer cells. Further, immunofluorescence experiments showed that RBBP8 knockdown reduced intranuclear RAD51 aggregation in HR repair induced by PARP inhibitors, while exogenous overexpression of the wild-type RBBP8 plasmid saved RAD51 activation (Figure 2A). These results show that RBBP8 not only participates in the G1/S phase transformation but also plays an important regulatory role in HR repair.

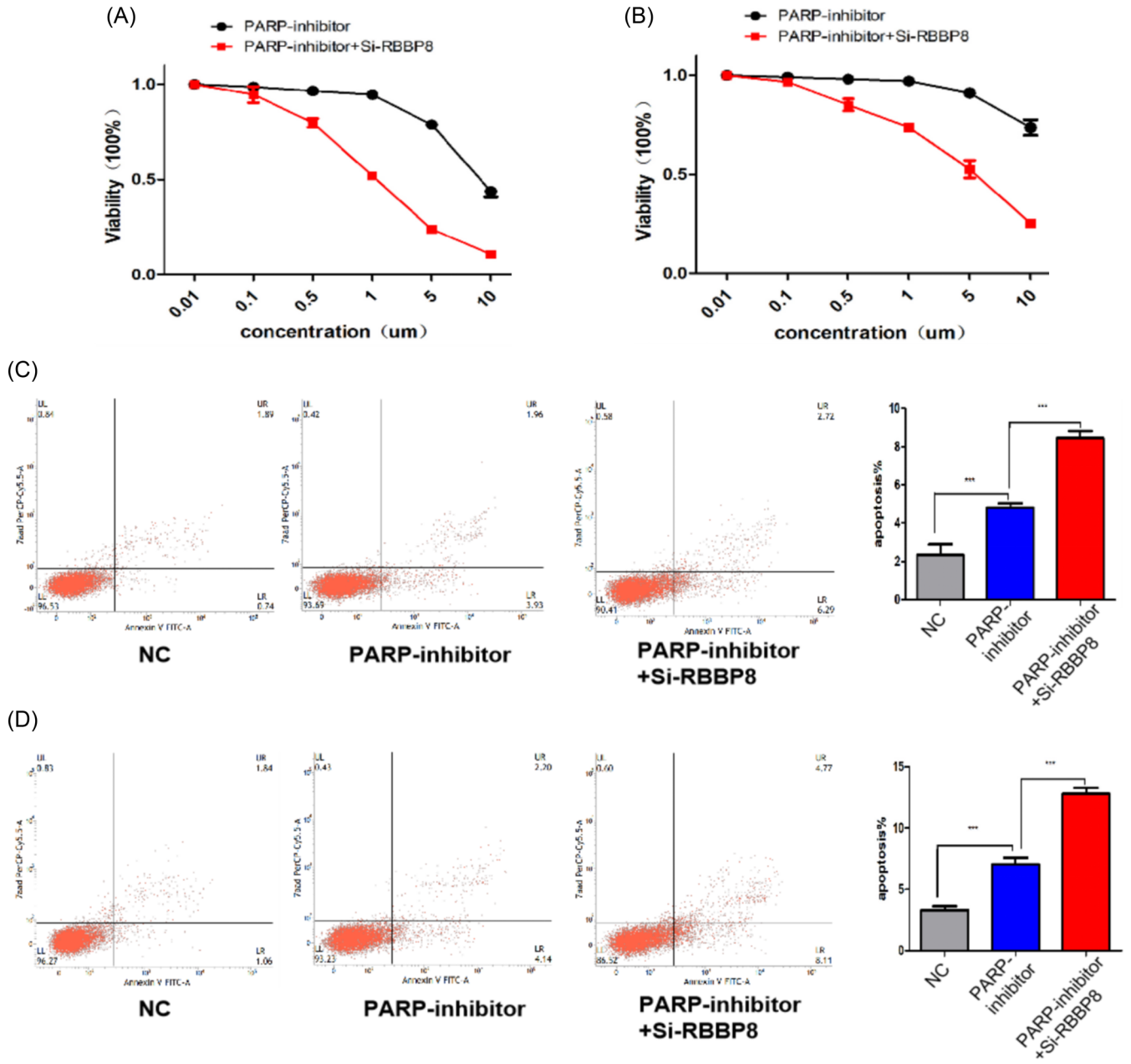

FIGURE 3 RBBP8 knockdown and PARP inhibitor treatment synergistically inhibited cell viability and promoted cell apoptosis in gastric cancer cell AGS (A, C) and N87 (B, D). All assays were performed in triplicate, and one representative result is displayed. ***p value < 0.001. PARP, poly ADP ribose polymerase.

Selecting the appropriate repair pathway at different stages of the cell cycle is of great significance for maintaining genetic integrity, and inappropriate HR repair initiation will also affect gene homeostasis.[19] However, the specific mechanism by which HR repair achieves its high cell cycle selectivity regulation (only in the S/G2 phase) is not yet clear. Previous studies have shown that the sequence around Ser327 of the RBBP8 protein belongs to the phosphorylation motif,[20] and CDK is the key phosphorylation protein of RBBP8.[21] In this study, we found that RBBP8 phosphorylation has high cycle selectivity consistent with HR repair (Figure 1C,D), suggesting that RBBP8 may induce an important switching effect in HR repair performance and G1/S transition. 5-bromodeoxyuridine (BrdU) is a thymidine nucleoside analog that reflects the formation of single-stranded DNA after terminal excision during DNA damage repair. Further immunofluorescence experiments proved that Si-RBBP8 knockdown inhibited intranuclear 5-bromodeoxyuridine staining at the DNA damage site (Figure 2B) under radiation injury. The terminal excision of double-stranded broken DNA is the key

initial stage of HR repair,[8] so we hypothesize that RBBP8 plays a role as a "switching protein" between the cell cycle and the HR repair opening: increased RBBP8 protein levels promote the G1/S phase transition and sister chromatid formation, which provide templates for HR repair; on the other hand, RBBP8 phosphorylation modification activates the DNA damaged repair complex to perform DNA terminal excision. Further elucidation of the role of the RBBP8-BRCA1 complex in the HR repair pathway is the next goal of this research group.

PARP inhibitors targeting the DNA damage repair pathway have recently become a hotspot in tumor treatment research. PARP inhibitors have proven effects on patients with breast and ovarian cancer with natural BRCA mutations (with HR repair defects), but they also have a narrow application scope and can induce drug resistance. In theory, the intervention of other key molecules in the HR repair pathway combined with PARP inhibitors could also achieve "synthetic lethality." Our study clarified that RBBP8 participates in DNA terminal excision in the DNA double-strand damage repair process. Through the cell phenotype experiment, we confirmed that the combination of RBBP8 molecular intervention and PARP inhibitor treatment has a synergistic inhibitory effect on cell viability and proapoptotic effect on gastric cancer cell lines (Figure 3A–D). Our team will further work on clarifying the mechanism of RBBP8 in the DNA damage complex and exploring the feasibility of a relevant small molecular inhibitor. It is of great clinical significance to find new strategies and new targets for the combination of PARP inhibitors to achieve synthetic lethal effects in non-BRCA naturally mutated gastric cancer.

AUTHOR CONTRIBUTIONS

Yang Yu and Yanhua Yin designed the work; Yang Yu and Shuxia Wang completed the experiment and drafted the manuscript; Guangsheng Wang revised and approved the final version.

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None.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The study protocol conformed to the standards set by the Declaration of Helsinki and was approved by the Ethics Committee of the Beijing Friendship Hospital, Capital Medical University.

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