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PRKDC is a prognostic marker for poor survival in gastric cancer patients and regulates DNA damage response

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ABSTRACT

A hallmark of gastric cancer is the high rate of genomic instability associated with deregulation of DNA damage repair pathways. DNA-Dependent Protein Kinase Catalytic Subunit (PRKDC) is a key component of the non-homologous end-joining (NHEJ) pathway. By reanalyzing transcriptome data of 80 pairs of gastric cancer tumors and the adjacent normal tissues from non-treated patients, we identified PRKDC as the top upregulated DNA damage repair genes in gastric cancer. High expression of PRKDC is associated with poor survival of gastric cancer patients, and genomic amplification of the gene is frequently observed across most gastric cancer subtypes. Knockdown of PRKDC in gastric cell lines resulted in reduced proliferation and cell cycle arrest. Furthermore, we showed that loss of PRKDC induced DNA damage and enhanced gastric cancer cell chemosensitivity to DNA-damaging reagents. Together, our results suggest that PRKDC is a prognostic marker of poor survival and is a putative target to overcome chemoresistance in gastric cancer.

1. Introduction

Gastric cancer is a malignancy with poor survival outcome and one of the leading causes of cancer-related mortality worldwide. Recent advances in tumor genetics have identified a number of genes highly associated with tumor initiation, progression and treatment response [2,13,16]. Particularly, a hallmark of gastric cancer is the high rate of genomic instability, which is associated with a greater propensity to accumulate DNA damage [6,11,14,15]. Upon DNA damage stimuli, cell cycle progression is suppressed by multiple checkpoint signaling pathways including the ATM/ATR−CHK1/CHK2–CDC25/WEE1 axis, leading to proliferation arrest and/or cell death. In tumor cells, however, DNA damage repair pathways are often disrupted to tolerate the high levels of DNA damage and/or to overcome cell cycle checkpoints [10,11,15,19,25]. As a result, errors during DNA synthesis or inaccurate repair are preserved and could finally lead to lesions that can give rise to gastric cancer. Deregulation of the DNA damage checkpoint genes and genomic instability are frequently observed in pre-cancerous lesions, indicating their potential roles as drivers for tumor imitation and progression. However, the molecular mechanisms underlying the changes of related protein groups during tumor progression and their effects on cell cycle and genome stability in gastric cancers remained largely unknown.

Dependent Protein Kinase Catalytic Subunit (PRKDC) is a key component of the non-homologous end-joining (NHEJ) pathway [3]. It functions as a scaffolding protein at DNA double-strand break (DSB) loci, and promotes several protein phosphorylation cascades that are essential to DNA damage response through its kinase activity [3,12,17]. Recently, there are growing evidence supporting the roles of PRKDC in regulating tumor progression and response to treatments [3,17], however, little is known regarding the expression and function of PRKDC in gastric cancer. In the current study, to identify new DNA damage repair proteins associated with gastric cancer, we reanalyzed transcriptome profiles of 80 gastric cancers and the paired normal gastric tissues, and identified PRKDC as one of the most significantly upregulated genes in gastric cancer. Furthermore, our integrative analysis revealed that high expression of PRKDC is a prognostic marker for poor survival in gastric cancer patients. In addition, genomic characterization of gastric cancer patients using TCGA data revealed PRKDC amplification across almost all the cancer subtypes. Together these data established PRKDC as a putative oncogene activated in gastric cancer. We reasoned that aberrant activation of PRKDC, probably driven by the underlying genetic

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portrait of the disease, may facilitate cell cycle progression and contribute to radio-and chemo-resistance of gastric cancer cells.

To determine the function of PRKDC on gastric cancer survival and cell cycle checkpoints regulation, we used siRNA-based approach to achieve efficient PRKDC knockdown in different gastric cancer cell lines. We also examined the roles of PRKDC in checkpoints regulation and DNA damage response. Moreover, we tested the efficacy of PRKDC knockdown on gastric cancer cell chemosensitivity to oxaliplatin-based chemotherapy, which induces DNA damage in the cells [18]. Our results suggested that PRKDC may predict clinical outcomes in gastric patients treated with DNA-damaging chemotherapy and PRKDC inhibition might be proven a novel approach to overcome chemoresistance in tumor cells.

2. Results

2.1. High expression of PRKDC associates with poor survival in gastric cancer patients

We reanalyzed transcriptome data of 80 pairs of gastric cancer tumors and the adjacent non-tumor tissues from 80 non-treated patients [5] (85% are of stage III and IV) to identify gene expression signatures highly expressed in tumors. The heatmap in Fig. 1A shows the expression patterns of the top 20 most significantly upregulated genes in gastric cancers as compared to their matched healthy control tissues. This analysis identified PRKDC as the top upregulated gene involved in DNA damage response in this patient cohort. To further determine the putative oncogenic function of PRKDC, we investigated the prognostic efficacy of PRKDC expression on the overall survival of gastric patients using data from the Kaplan–Meier plotter online database [7], which contain gene expression profiles and clinical information from 876 gastric cancer patients (containing both treated and untreated). These patients were divided into two groups associated with high (n = 365) and low (n = 511) PRKDC expression using the median gene expression as a cutoff. As shown in the Kaplan–Meier survival plots (Fig. 1B), high PRKDC expression was associated with a significantly shorter overall survival in these gastric cancer patients. The results remained robust limiting the analysis to 380 untreated patients, such that high expression of PRKDC is associated with poor survival of gastric patients (Figure S1). Consistent with our results, elevated PRKDC expression has been reported in multiple cancer types such as breast cancer [22] and hepatocellular carcinoma [4], while high PRKDC expression was associated with higher tumor grade and unfavorable outcomes (see discussion). Our results thus established PRKDC as a putative oncogene and prognosis marker in gastric cancer.

2.2. Frequent copy number gain of PRKDC in gastric cancer

Alternations in gene expression is frequently associated with copy number variations in tumor cells [26]. In light of this, we examined PRKDC copy numbers in TCGA gastric cancer datasets containing over 300 gastric cancer and normal sample pairs of seven major gastric cancer subtypes, including diffuse gastric adenocarcinoma, gastric adenocarcinoma, gastric intestinal type adenocarcinoma, gastric papillary adenocarcinoma, gastric tubular adenocarcinoma, mucinous gastric adenocarcinoma and signet ring cell gastric adenocarcinoma. As shown in Fig. 2, our results showed copy number gains of PRKDC in all seven gastric cancer subtypes, with the most dramatic amplification observed in patients with gastric intestinal type adenocarcinoma and gastric papillary adenocarcinoma. As a control, we also examined copy number variation of a housekeeping gene GAPDH (Fig. 2). In striking contrast to PRKDC, the copy number of GAPHD was not changed in any of the gastric cancers, indicating the genomic amplification was specific to PRKDC loci. We thus suggest that the elevated levels of PRKDC in gastric cancer was at least in part caused by the change in copy number.

2.3. Depletion of PRKDC leads to proliferation inhibition and mitotic arrest

In light of the above results, we hypothesized that PRKDC might play a critical role to facilitate gastric cancer proliferation and survival. To investigate the regulatory roles of PRKDC in gastric cancer cells, we used a siRNA-based approach to knockdown PRKDC in two different gastric cancer cell lines, BGC-823 and NCL-N87. As shown in Fig. 3A, western blot revealed robust PRKDC protein depletion in both cell lines upon PRKDC knockdown. PRKDC knockdown resulted in significant proliferation inhibition in both cell lines (Fig. 3B), supporting the oncogenic role of PRKDC in promoting gastric cancer progression. Consistent with proliferation inhibition, as shown in Fig. 3C-D, PRKDC knockdown in BGC-823 cells resulted in a significant increase of cells arrested in G2/M phase (21.0% in PRKDC knockdown *vs.*9.2% in control knockdown, $p < 0.001$). As shown in Fig. 3E-F, similar results were observed in PRKDC knockdown in NCL-N87 cells (18.3% in PRKDC knockdown *vs.*7.1% in control knockdown, $p < 0.001$), indicating mitotic arrest being a major defect upon PRKDC depletion that might lead to cell proliferation arrest. This is in line with previous studies that PRKDC plays an important role in coordinating chromosomal segregation and microtubule dynamics [1,9,12]. Furthermore, to rule out off-target effect, we have designed another siRNA targeting PRKDC and performed the proliferation and cell cycle analyses after transfection. As shown in Figure S2, knockdown of PRKDC with the independent siRNA also resulted in significant proliferation inhibition and cell cycle arrest.

2.4. Depletion of PRKDC induces DNA damage and sensitizes gastric cancer cells to chemotherapies

Given the putative role PRKDC plays in double-strand break repair, we hypothesized that gastric cancer cells lacking PRKDC might exhibit deficient DNA damage repair and accumulation of damages. To this end, DNA damages in PRKDC knockdown cells were examined by

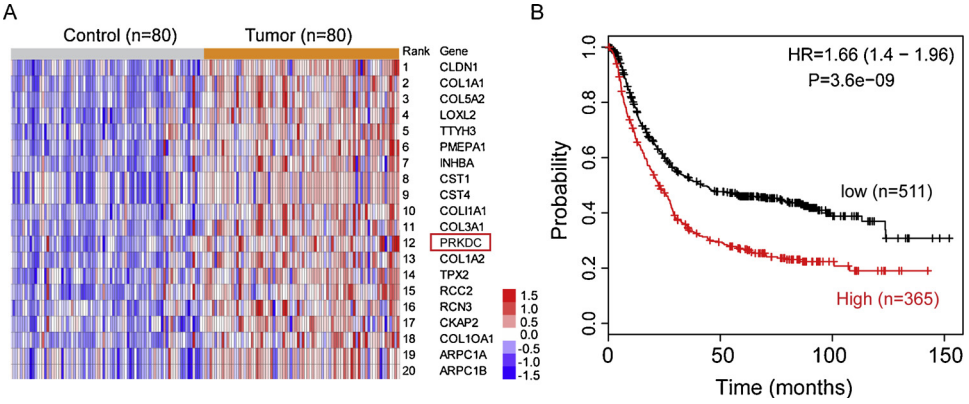

Fig. 1. High expression of PRKDC associates with poor survival in gastric cancer patients. (A) The heatmap shows the expression of top 20 most significantly upregulated genes in 80 gastric cancer tumors *versus* the paired non-tumor tissues. Gene expression was normalized by mean of all samples, samples with relative high expression of a given gene is marked with red and those with low expression were marked blue. (B) Kaplan–Meier plots show overall survival of patients with relatively high or low expression of PRKDC. High or low expression was defined by median.

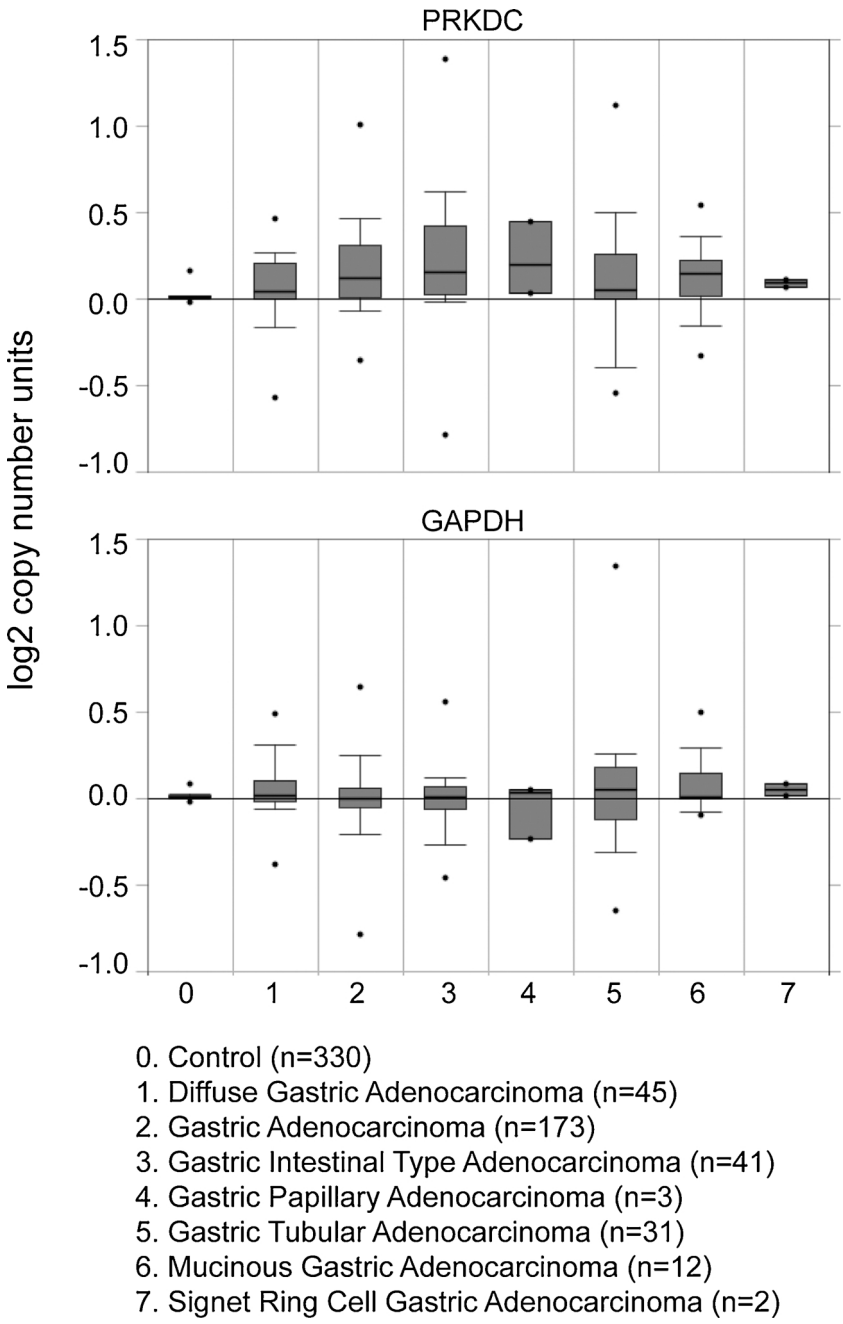

Fig. 2. Frequent copy number gain of PRKDC gene in different gastric cancer subtypes. Relative copy number of PRKDC and GAPDH in control and different gastric cancer subtypes are shown (log_2 copy number units, diploid is shown as 0). Center lines show the means, box limits indicate the 25th and75th percentiles, and whiskers extend to minimum and maximum values. Data present mean ± SEM of all samples.

immunofluorescence staining of DNA damage marker Phospho-Histone H2A.X (γ-H2A.X). As shown in Fig. 4A, γ-H2A.X signaling was barely observed in control knockdown cells. In contrast, PRKDC depletion resulted in robust γ-H2A.X accumulation in both BGC-823 and NCL-N87 gastric cancer cell lines. This result is further validated by western blot such that γ-H2A.X expression was significantly increased in PRKDC knockdown cells compared to control knockdown (Fig. 4B).

In light of its critical role in DNA damage response, overexpression of PRKDC might be associated with chemoresistance of gastric cancer cells. Consequently, PRKDC depletion might thus sensitize gastric cancer cells to DNA-damaging reagents, a conception important to exploit the benefit of PRKDC inhibition in sensitization of radio- and chemo-therapies [19]. Oxaliplatin, a non-nephrotoxic platinum analog, induces DNA damage in cancer cells and has been recently proven to be an effective first-line chemotherapy for advanced gastric cancer patients [18]. We thus examined the efficacy of PRKDC knockdown on gastric cancer cell response to oxaliplatin treatment. As shown in Fig. 4C, BGC-823 and NCL-N87 cells with control or PRKDC knockdown were treated with different doses of oxaliplatin for 48 h, and the cell survival were examined by CellTiter-Glo assay and normalized by control cells treated with DMSO. Our results showed that PRKDC depletion resulted in significantly reduced chemoresistance in both cell lines. Consistent with our results, previous study showed that PRKDC plays a critical role in acquired platinum resistance [21]. We thus suggest that combination chemotherapy with PRKDC inhibitor and oxaliplatin might be a potentially promising regimen to overcome drug resistance in gastric cancer.

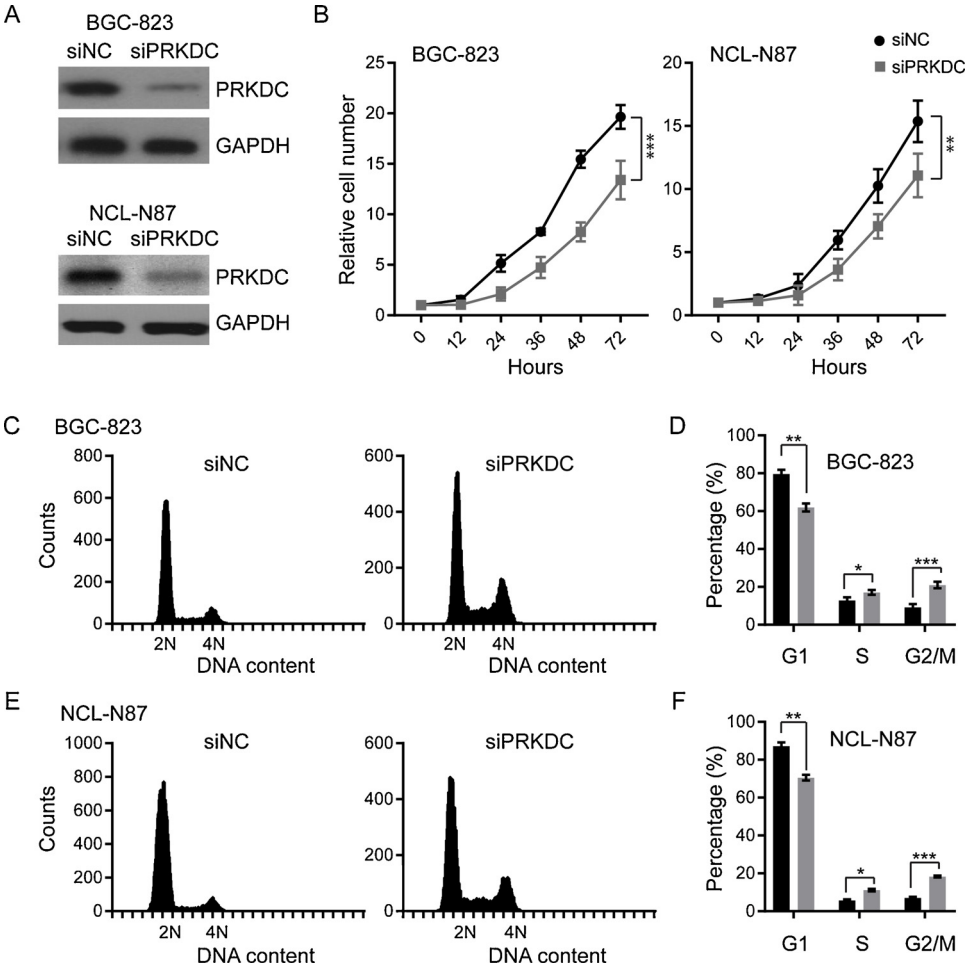

Fig. 3. Depletion of PRKDC leads to proliferation inhibition and mitotic arrest. (A) Western blot of PRKDC expression in different gastric cancer cell lines transfected with control or PRKDC siRNAs for 24 h. (B) Proliferation curves of different gastric cancer cell lines transfected with control or PRKDC siRNAs are shown. (C) BGC-823 cells were transfected with control or PRKDC siRNAs for 24 h and then stained with PI for analysis of cell cycle distribution. (D) Proportions of cells at different cell cycle phases in (C) are shown. Similar, results of NCL-N87 cells are shown in (E) and (F). Data present mean ± SEM of three replicates. ***P < 0.001; **P < 0.01; *P < 0.05.

3. Discussion

The initiation and development of gastric cancer is a multi-stage process involving a number of genetic lesions and aberrant signaling regulation, identifying key molecules. Characterization of biological processes critical to gastric cancer tumorigenesis would facilitate the development of novel diagnostic/prognostic biomarkers and novel therapies. In the current study, through integrative analysis of transcriptome, copy number and clinical data of gastric cancer patient, we identified PRKDC as a putative oncogene for the disease. Consistent with these results, marked proliferation inhibition and G2 phase cell cycle arrest were observed after PRKDC knockdown in gastric cancer cell lines. In addition, our results showed that PRKDC depletion induced DNA damage and sensitized gastric cancer cells to oxaliplatin. We thus suggested that high PRKDC expression is a putative prognostic biomarker for poor survival in gastric cancer patients, and selective inhibition of PRKDC may provide a new therapeutic approach for gastric cancer treatment.

Consistent with our results, overexpression of PRKDC has been identified in several cancer types and was associated with advanced clinical stages and/or unfavorable outcomes. For example, previous studies showed elevated PRKDC expression in breast cancers compared to normal tissues, and high expression of the gene was significantly correlated with advanced tumor grade, lymph node metastasis, as well as poor patient survival [22]. Moreover, it has been reported that high expression of PRKDC was associated with advanced clinical stage and metastasis in non-small cell lung cancer [24]. Increased PRKDC expression was observed in high-grade lymphoma lymph node samples *versus* low-grade lymphoma patients and had also been found to be correlated with poor clinic outcomes [8]. Increased PRKDC expression was also observed in liver cancer, and was associated with treatment-resistance and faster progression [4]. Consistently, our results and others identified strongly correlated tendency between increased PRKDC expression and tumorigenesis in multiple different cancer types, in line with the notion that PRKDC protein expression may serve as a prognostic predictor.

Of note, there are several other important regulatory effects by PRKDC beyond DNA damage repair and cell cycle regulation that might also facilitate tumorigenesis. The precise cellular mechanisms through which loss of PRKDC induces DNA damage are currently unknown. However, it is well-established that accumulation of DNA damage marker (γH2AX formation) and hypersensitivity to irradiation occur in multiple cancer cell lines upon PRKDC loss, probably as a result of impaired DNA repair or attenuated DNA damage signaling transduction regulated by PRKDC. These could result in increased DNA damage leading to proliferation arrest and cell death. As a result, inhibition of PRKDC has proven to be an efficient approach to sensitize human cancer cells and tumor xenografts to ionizing radiation and DNA damage reagents, highly implicated in overcoming therapeutic resistance. Due to its serine/threonine-protein phosphorylation kinase activity, it is possible that PRKDC might mediate a broad signaling pathways involved in, for example, migration and invasion, to facilitate tumor metastasis [4,24]. There is also evidence suggesting that PRKDC is a key regulator of transcription *via* modulating phosphorylation of a number of known oncogenic transcription factors such as JUN, FOS, OCT-1 and SP1 [20]. We thus highlight the potential of PRKDC as a drug target to develop novel therapeutic option against gastric cancer. Pharmacological inhibition of PRKDC might be to overcome chemoresistance in

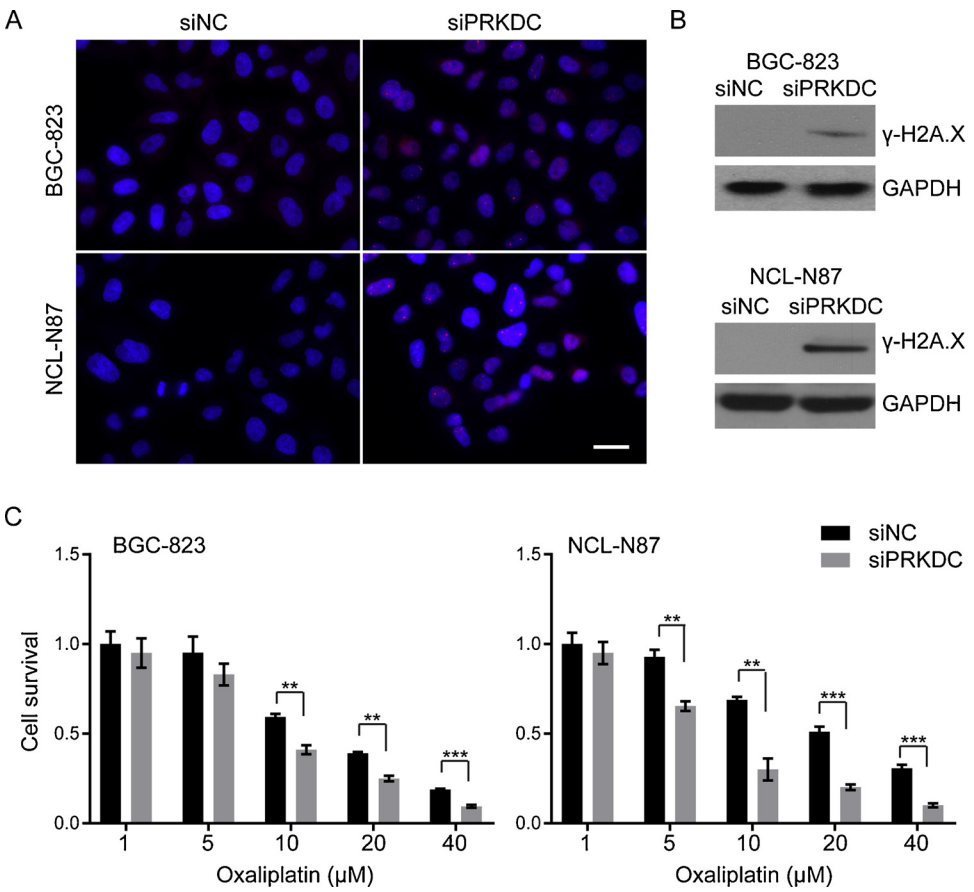

Fig. 4. Depletion of PRKDC induces DNA damage and sensitizes gastric cancer cells to chemotherapies. (A) Fluorescent staining of γ-H2A.X in cells transfected with control or PRKDC siRNAs. Scale bar: 25 μm. (B) Western blot of γ-H2A.X in cells transfected with control or PRKDC siRNAs. (C) Relative survival of cells transfected with control or PRKDC siRNAs treated with different doses of oxaliplatin. Data were normalized relative to the mean value of control knockdown treated with DMSO (0 μM oxaliplatin). Data present mean ± SEM of three replicates. ***P < 0.001; **P < 0.01; *P < 0.05.

cancer cells [18], combined chemotherapy with PRKDC inhibitor and oxaliplatin might enhance the tolerance and reduce the required chemoresistance in gastric cancer patients. A deeper understanding about how PRKDC overexpression is correlated with cancer susceptibility and drug resistance would help to identify patients who would most benefit from PRKDC inhibition.

4. Materials and methods

4.1. Integrative analysis of gastric cancer

Transcriptome data of 160 samples were analyzed on parried tumor and adjacent non-tumor tissues from 80 gastric cancer patients were obtained from GEO database (GSE27342) and subsequently analyzed using the Oncomine (www.oncomine.org). Copy number variations were obtained from Stomach Adenocarcinoma DNA Copy Number Dataset (http://gdac.broadinstitute.org/runs/stddata_2013_05_23/data/.) The DNA samples of gastric tumor and normal controls were originally run on the Affymetrix SNP 6.0 platform for DNA copy number analysis, and processed data were obtained from Oncomine database. The predictive value of PRKDC on overall survival of gastric cancer patients were analyzed using the Kaplan–Meier plotter online database (www.kmplot.com) [23].

4.2. Cell culture

Gastric cancer cell lines BGC-823 and NCL-N87 were obtained from Shanghai Institute of Cell Bank (Shanghai, China). Cells were cultured in RPMI-1640 mediam (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco, Gaithersburg, MD, USA) in an incubator of 5% CO2 at 37 °C.

4.3. Cell viability assay

Cell viability assay was performed using CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI, USA) as per manufacturer's instructions. Briefly, cells were seeded at 5000 cells/well in a 96-well plate and treated as described in the figures, with three biological replicates for each group. After treatment, 100 μl of CellTiter-Glo® reagent were added to each well is added to each well and incubated at 37 °C to allow cells to convert resazurin to resorufin. The fluorescent signal is read using a fluorescence GloMax™ 96 Microplate Luminometer (Promega, Madison, WI, USA).

4.4. Cell cycle analysis

Cell-cycle analysis was conducted using a FACSCalibur flow cytometer (BD Biosciences, Woburn, MA, USA). Cells were fixed in 75% ethanol at 4 °C overnight, washed twice with PBS, and then incubated with PI solution (Promega, Madison, WI, USA) for 30 min as per manufacturer's instructions. The percentage of G1, S and G2/M cells was quantified using FlowJo 7.0 software.

4.5. Small interfering RNA mediated knockdown

Gastric cancer cells were seeded overnight at a density of 5000 cells/well in a 96-well plate or 2×10^4 cells/well in a 24-well plate. siRNA targeting PRKDC or control siRNA were obtained from Dharmacon SMARTpool (Lafayette, CO, USA; catalogue no. D-005030-01 for PRKDC and D-001210-01-05 for control, respectively). In addition, we designed another set of siRNAs targeting PRKDC 5′-GAAAGG AGGTTCTAAACTAC

T-3′, or control 5′-GCTACGTGAATATAGACCATA-3′. Cells were transfected using Lipofectamine 2000 (Invitrogen) in an OptiMEM

serum-free medium (Thermo Fisher Scientific, Waltham, MA, USA) as per manufacturer's instructions.

4.6. Western blot

The following primary antibodies were used in western blot: anti-PRKDC (1:1000, Cell Signaling, Danvers, MA, USA), anti-γH2AX (1:2000, Cell Signaling, Danvers, MA, USA), anti-GAPDH (1:5000, Sigma-Aldrich, St. Louis, MO, USA). For immunoblots, whole cell lysates were prepared using RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA) and 10 μg protein per sample were separated by SDS page, and transferred to a PVDF membrane (Millipore, Bedford, MA, USA). Proteins were incubated with primary antibodies at 4 °C overnight and horseradish peroxidase-conjugated secondary antibodies for one hour at room temperature.

4.7. Immunofluorescence staining

The cells were seeded in a 24-well plate at a density of 2×10^4 cells/well and transfected with PRKDC or control siRNA for 24 h. Cells were then fixed with 4% paraformaldehyde for 15 min, and then treated with 0.1% Triton X-100 for 15 min at room temperature. Subsequently, the cells were incubated with anti-γH2A.X antibody (Cell Signaling, Danvers, MA, USA) in the presence of 1% BSA at room temperature for 1 h. Subsequently, the cells were washed 3 × 5 min with PBST, and incubated at room temperature for 1 h with cy3-labeled anti-IgG antibody (Cell Signaling, Danvers, MA, USA) in the presence of 1% BSA. Cells were washed 3 × 5 min with PBST and nuclei were stained with DAPI.

4.8. Statistical analysis

Statistical analysis was performed using PRISM 6 (GraphPad, CA, USA). The significance of differences between groups were determined using Student's *t*-test Differences were considered to be significant with $P < 0.05$. Data present mean ± SEM of three replicates. Survival curves were constructed with the Kaplan-Meier method and compared using log-rank test. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

Author contributions

YZ, WY, GW and YD designed the experiments. YZ and WY performed the integrative bioinformatics analysis, GW, CW, ZJ, DZ performed the knockout and western blot. YZ performed immunefluorescent staining. WY, GL and XW performed cell cycle analysis. MT, YL, YZ helped with cell culture. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.prp.2019.152509.

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