

## Silencing of CHFR Sensitizes Gastric Carcinoma to PARP Inhibitor Treatment



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### Abstract

CHFR is a tumor suppressor that not only recognizes poly(ADP-ribosylation) (PARylation) signals at the sites of DNA damage but also is downregulated in many types of cancer. However, the underlying mechanism linking its role in PARylation-mediated DNA damage repair and tumor suppression is unclear. Here, we examined a panel of gastric cancer cell lines as well as primary tissue samples from gastric cancer patients, and found that CHFR expression was silenced by DNA hypermethylation in gastric cancer including 38.46% of primary gastric cancers. DNMT1 was associated with aberrant methylation of CHFR, and the expression of CHFR was restored by DNMT1 inhibitor 5-aza-2-deoxycytidine (5-aza-CdR) treatment. Moreover, we found that loss of CHFR abolished DNA damage repair and sensitized gastric tumor cells to PARP inhibitor treatment. Thus, our study reveals a potential therapeutic approach for treating gastric cancer with PARP inhibitor and lacking CHFR can serve as a biomarker for predicting the efficacy of PARP inhibitor on the gastric tumor treatment in future.

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### Introduction

Gastric cancer is the fourth most common cancer and a major leading cause of cancer-related deaths worldwide [1–3]. The five-year survival rate for GC patients remains in a low level of around 37% [4]. Aberrant epigenetic modification has been increasingly found as an important contributing factor in cancer development [5,6]. DNA

methylation is a major epigenetic modification involving the addition of a methyl group to the 5 position of cytosine by DNA methyltransferase (DNMT) to form 5-methylcytosine (5-mC). DNA hypermethylation at promoter regions induces transcription inactivation of tumor suppressor genes [7]. Such aberrant methylation has been detected frequently in gastric cancer [8]. And one of the targets of DNA hypermethylation is CHFR (checkpoint protein with FHA and RING finger domains).

CHFR is a nuclear polypeptide with an N-terminal FHA domain, a central RING finger domain acting as an ubiquitin E3 ligase and a C-terminal cysteine-rich region [9]. Recently, a poly-ADP ribose binding zinc-finger (PBZ) motif was identified in the C-terminal region of CHFR [10], which was shown to mediate a protein–protein interaction with PARP-1 and recognized poly(ADP-ribose) (PAR) [11,12]. The interaction between PARP1 and CHFR is functionally important, which not only allows CHFR to be recruited to areas of DNA damage [13] but also through CHFR-mediated ubiquitination of PARP-1 and its subsequent

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proteasomal degradation, it removes PARP-1 from damaged chromatin once the DNA repair machinery has been initiated [14].

In the present study, we examined the methylation status of *CHFR* gene and the expression of *CHFR* mRNA and protein in gastric cancer. We found that loss of *CHFR* widely occurred in gastric cancer. Moreover, lacking the expression of *CHFR* was associated with promoter hypermethylation and the recruitment of DNMT1 to the promoter. Moreover, gastric cancer cells lacking *CHFR* had DNA damage repair defects and were hypersensitive to PARP inhibitor treatment. Collectively, our study may reveal PARP inhibitor treatment as a potential therapeutic strategy for treating gastric cancer lacking the expression of *CHFR*.

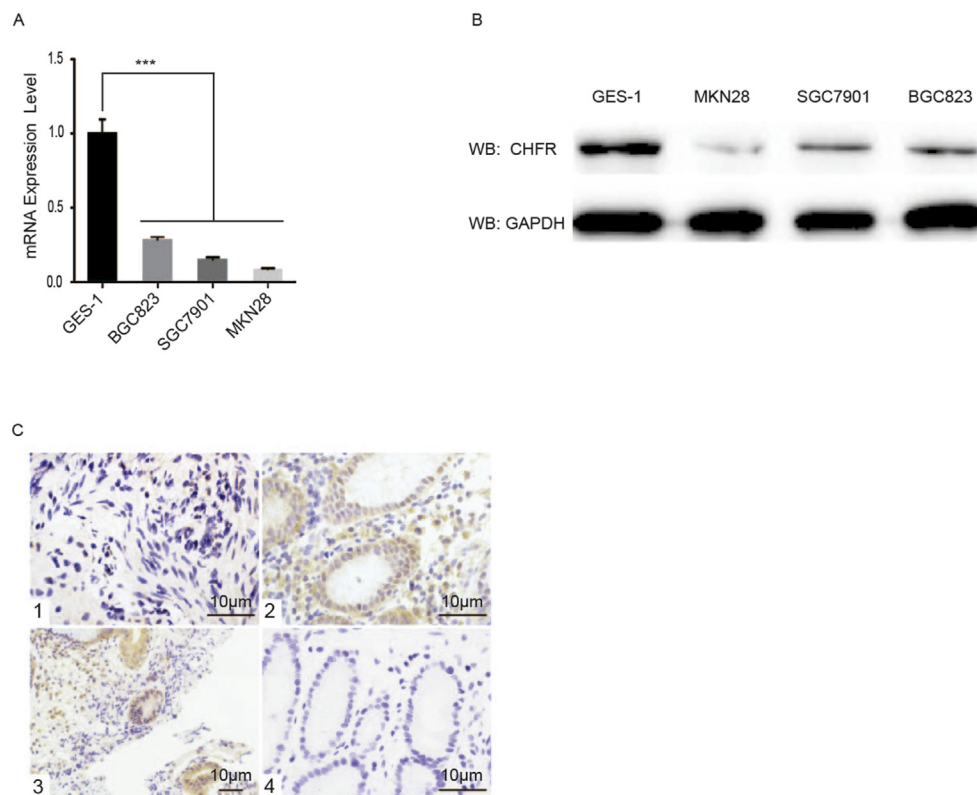
## Results

### Loss of *CHFR* Expression in Gastric Cancers

To carefully examine the status of *CHFR* in gastric cancer, we analyzed *CHFR* gene transcription in human gastric cancer cell lines including SGC7901, MKN28, and BGC823 as well as normal gastric cell GES-1. Quantitative PCR reveals that *CHFR* expression levels in three gastric cancer cell lines are significantly lower than that in GES-1 (Figure 1A). We also performed western blotting to assess *CHFR* expression in above-mentioned cells lines. Consistently, our

result shows that *CHFR* protein expression is much lower in gastric cancer cells (Figure 1B).

Next, we examined *CHFR*'s expression in normal gastric tissues and primary gastric carcinomas. Total 52 samples including 26 gastric cancer samples and 26 adjacent normal tissues were collected from Peking University Third Hospital. The immunostaining intensity for each sample was scored with following criteria: 0, for 0–10% stained tissue; 1, for samples stained between 10 and 40% of tissue; 2, for samples stained between 40 and 80% of tissue; and 3, for samples with more than 80% of stained tissue. Signal intensity was scored as strong (3, dark brown color), moderate (2, medium brown color), weak (1, light brown color), or null (0, no immunostaining). Total immunostaining score results from multiplication of both parameters, and was classified as follows: strong (+++, total immunostaining score = 6–9), moderate (++), total immunostaining score = 3–4), weak (+), total immunostaining score = 1–2), and null (-, total immunostaining score = 0). Again, with immunohistochemistry staining, we found that *CHFR* expression was prominent in the normal gastric gland epithelia tissue, but rarely existed in gastric cancer tissues (Figure 1C, Table 1). Among the 26 normal stomach tissue samples, 22 of them (84.62%) are positively stained with anti-*CHFR* antibody and strong and moderate staining are 18 samples (69.23%). However, in 26 tumor tissues, 10 of them (38.46%) show negative *CHFR* staining and 14 of them (53.85%) showed weak *CHFR*



**Figure 1. The expression of *CHFR* in gastric cancer.** (A) Analysis of *CHFR* expression in gastric cancer cell lines (BGC823, SGC7901, MKN28) and normal stomach mucosa cells (GES-1) using RT-qPCR. GAPDH was used as a control. (B) Western blot analysis on the expression of *CHFR*. Cell lysates were examined by anti-*CHFR* antibody. GAPDH was used as the loading control (bottom panel). (C) Immunohistochemistry using anti-*CHFR* antibody shows the prominent staining in the gastric gland epithelia of normal primary gastric samples but not in gastric cancer samples. 1. Cancer tissue; 2. Normal tissue; 3. Cancer and surrounding normal tissue; and 4. Nonspecific immunoglobulin was used as a negative control. Magnification: 1, 2 and 4  $\times$  400; 3  $\times$  100. (To show the immunostaining profile of carcinomas and normal tissue surrounding carcinomas in the same microscopic field, 100 $\times$  magnification was used).

**Table 1.** CHFR and 5-mC levels in gastric cancer tissues and adjacent normal tissues

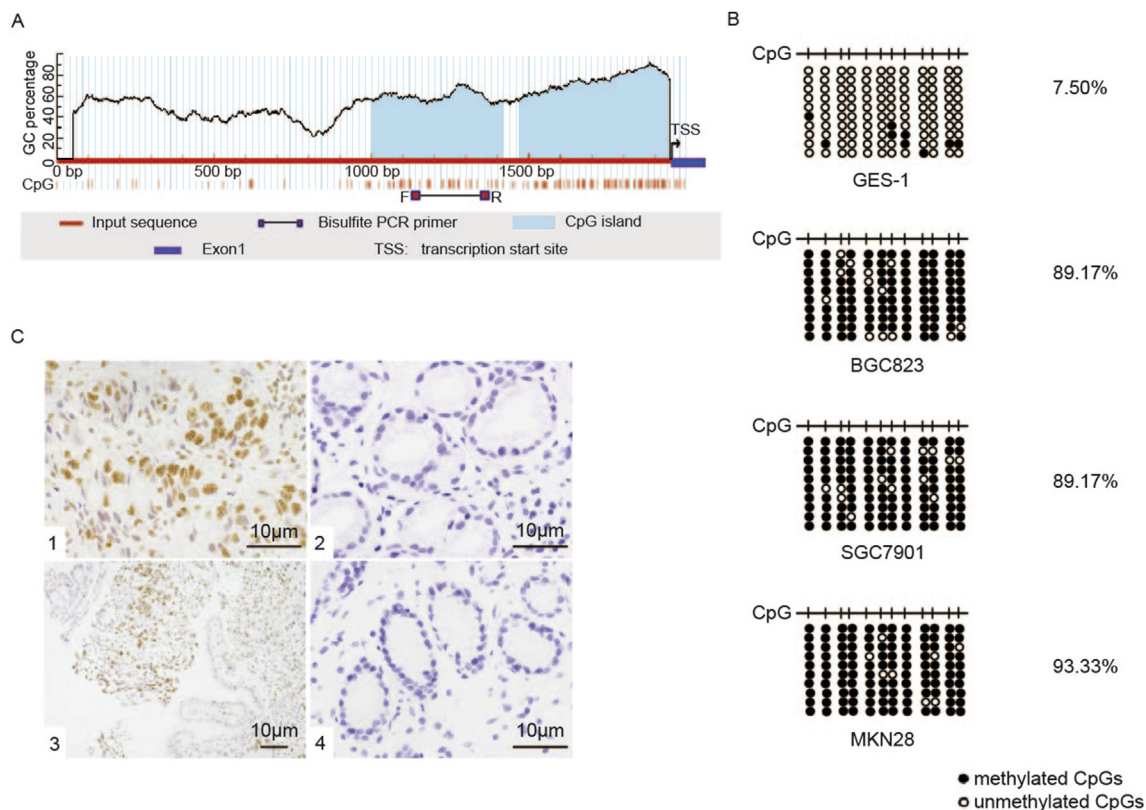
		positive			Negative	P*
		Strong	Moderate	Weak		
Cancer Tissues	CHFR	0(0%)	2(7.69%)	14(53.85%)	10(38.46%)	0.00058
	5-mC	6(23.08%)	4(15.38%)	16(61.54%)	0(0%)	
Normal Tissues	CHFR	4(15.38%)	12(46.15%)	6(23.08%)	4(15.38%)	0.032
	5-mC	0(0%)	4(15.38%)	8(30.77%)	14(53.85%)	

expression (Figure 1C). Collectively, there is a significant correlation between loss of CHFR expression and gastric cancer.

**Aberrant DNA Methylation is Associated with CHFR Silencing**

Our analysis on CHFR promoter region reveals typical CpG islands (Figure 2A), which can be hypermethylated for silencing gene expression. We examined DNA methylation status at the CpG islands in gastric cancers using bisulfite sequencing with primers close to the transcription start site of CHFR gene (Figure 2A). Aberrant promoter hypermethylation of CHFR was detected in all three gastric cancer cell lines, in which the gene was almost silenced (Figure 2B). In contrast, little DNA methylation was found in these CpG islands in the normal cells, suggesting that silencing CHFR by promoter hypermethylation

is associated with gastric tumorigenesis. In addition, we detected the global methylation status in above-mentioned 52 tissue samples by immunohistochemistry using anti-5mC antibody. Our results showed that there was an obvious difference in staining intensity between carcinomatous zones and normal tissues in the paired samples from the same patient (Figure 2C). Among these primary tissue samples, 26/26 (100%) cancer tissues were 5 mC positive, whereas only 12/26 (46.15%) normal tissues were 5-mC positive, including 4 samples with moderate 5 mC staining, 8 samples with weak 5 mC staining, and the rest 14 normal tissues were 5 mC negative (53.85%). Therefore, the 5 mC immunostaining intensity in gastric cancer tissues is significantly higher than that in normal tissues, which is well anticorrelated with the expression CHFR (Table 1).



**Figure 2. Analysis of the promoter methylation of CHFR in gastric cancer.** (A) A diagram of the CpG islands of CHFR. The CpG sites are indicated by vertical bars; the arrow indicates the transcription start site. The primers used for bisulfite sequencing analysis are shown by the rectangle. The region analyzed by bisulfite sequencing is indicated with a bar. (B) Bisulfite sequencing analysis of the CpG islands. The PCR products were cloned into pMD-19T, and at least 10 clones from each cell line were sequenced. Open and closed areas represent unmethylated and methylated CpG dinucleotides, respectively. Cell lines are shown. (C) Representative images of 5 mC in malignant and normal gastric tissue samples. 1. Cancer tissue; 2. Normal tissue; 3. Cancer and surrounding normal tissue; and 4. Nonspecific immunoglobulin was used as a negative control. Magnification: 1,2 and 4 × 400; 3 × 100. (To show the immunostaining profile of carcinomas and normal tissue surrounding carcinomas in the same microscopic field, 100× magnification was used).

### *DNMT1 is Associated with Aberrant DNA Methylation at the Promoter of CHFR*

To further explore the molecular mechanism of DNA hypermethylation in silencing CHFR expression, we examined if DNMT inhibitor treatment could reactivate gene transcription of *CHFR*. Gastric cancer cells were treated with 5  $\mu$ M or 10  $\mu$ M 5-aza-2-deoxycytidine (5-aza-CdR). The expression of CHFR in gastric cancer cells was markedly restored (Figure 3A). Bisulfite sequencing analyses reveal that the level of DNA methylation at *CHFR* gene promoter was suppressed following 5-aza-CdR treatment (Figure 3B). ChIP assays with anti-DNMT1 antibody show that DNMT1 is associated with promoter region of CHFR surrounding the CpG islands in gastric tumor cells including BGC823, SGC7901, MKN28, but not in the control normal cells GES1 (Figure 3C), suggesting that DNMT1 is responsible for DNA hypermethylation and *CHFR* gene silencing.

### *Loss of CHFR Causes DNA Damage Repair Defects*

Because CHFR recognizes PARylation signals at DNA lesions, we ask if CHFR is involved in DNA damage repair. We treated gastric cancer cells lines and normal cell line with 5 mM methyl methanesulfonate (MMS) to induce DNA lesions including DNA single- and double-strand breaks, and measured DNA damage repair kinetics with the comet assay under alkaline condition. As shown in Figure 4A, in a time course of repair assays, we found that DNA lesions were repaired in GES-1 cells that are expressing CHFR. In contrast, DNA damage repair is significantly delayed in gastric tumor cells including SGC7901, BGC823, and MKN28, which were not expressing CHFR. Moreover, restoration of CHFR expression by 5-aza-CdR treatment rescued DNA damage repair in SGC7901, BGC823, and MKN28 cells (Figure 4B), suggesting that lacking the expression of CHFR in gastric tumor cells impairs DNA damage repair.

### *Loss of CHFR Sensitizes Gastric Tumor Cells to PARP Inhibitor Treatment*

Accumulated evidence suggests that PARP inhibitor treatment selectively kills tumor cells with DNA damage repair defects. Because CHFR recognizes PARylation signals and participates in DNA damage repair, we then further evaluate the role of CHFR on PARP inhibitor treatment on gastric cancer. We treated gastric cancer cells with Olaparib (5  $\mu$ M), a potent PARP inhibitor, for a short-term apoptosis assay. We found that apoptosis significantly occurred in gastric cancer cells including BGC823, SGC7901, and MKN28, but not in control normal GES-1 cells (Figure 5A), suggesting that CHFR expression correlates specifically with cellular sensitivity to PARP1 inhibitor treatment. To further validate the results in long-term cell viability assays, we performed colony formation assays. With 1  $\mu$ M Olaparib, gastric cancer cells without CHFR expression formed significant less cell colonies compared with the mock treatment (Figure 5B). In contrast, the growth of normal GES-1 cells was not suppressed by the PARP inhibitor treatment (Figure 5B). Collectively, these results suggest that loss of CHFR expression sensitizes gastric tumor cells to PARP1 inhibitor treatment.

### **Discussion**

CHFR is a tumor suppressor [15,16] and is frequently silenced by promoter methylation in various types of tumor [9,16,17]. Here, we provide clear evidence that DNA hypermethylation at the promoter region of *CHFR* gene frequently shut down its expression in gastric cancer. The immunohistochemistry results from paired gastric cancer

samples and adjacent normal tissue samples further confirmed that silencing of *CHFR* methylation occurred in gastric carcinoma tissues. The DNMT inhibitor 5-aza-CdR treatment restored the expression of CHFR, indicating the transcriptional silencing of CHFR is caused by DNMT-mediated DNA hypermethylation. The ChIP analyses showed that DNMT1 is recruited to the hypermethylated promoter, suggesting that it plays a key role in the methylation-dependent silencing of CHFR. Thus, the molecular mechanism of CHFR silencing is clearly demonstrated in our studies.

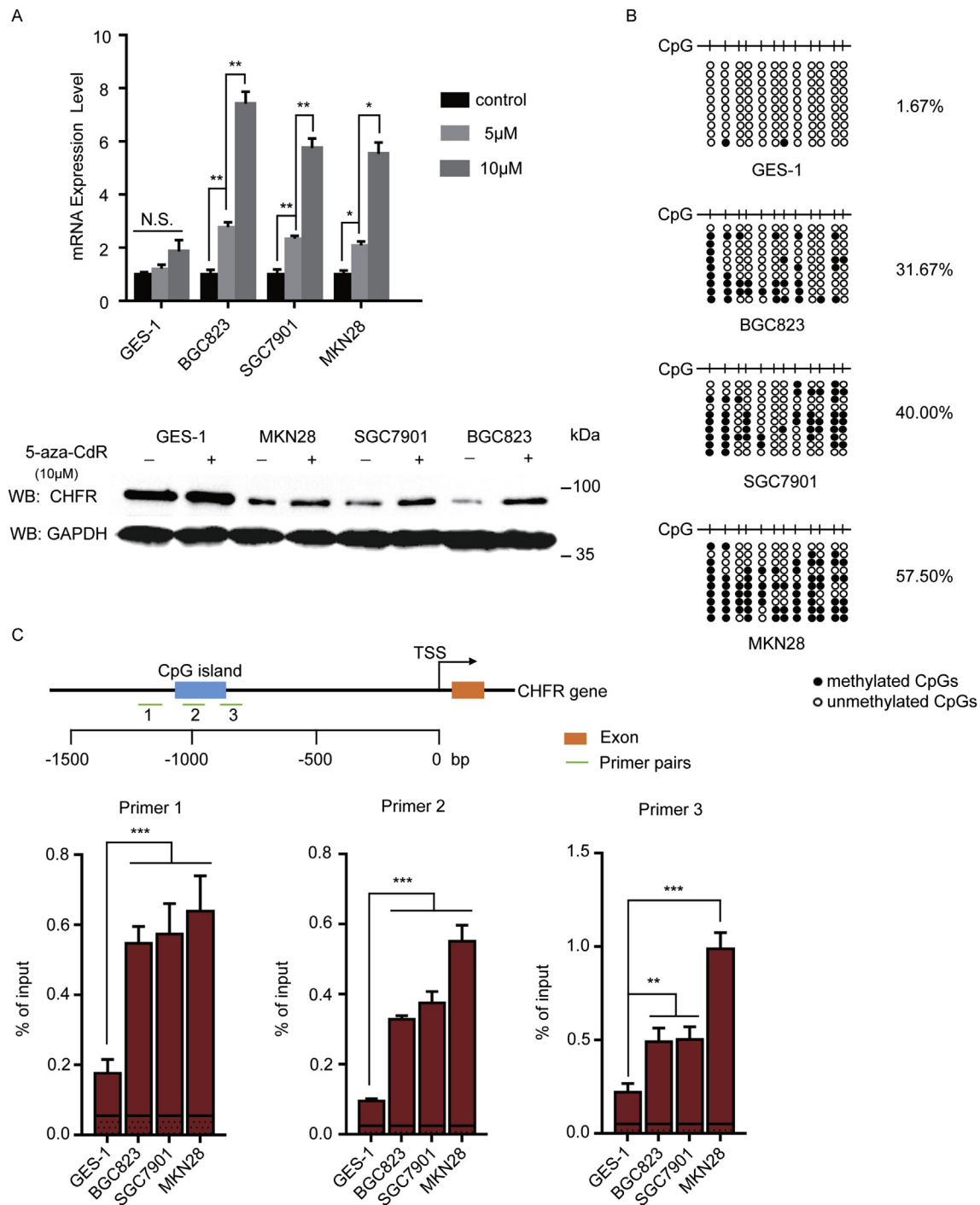
Moreover, we found that lacking of CHFR expression was associated with DNA damage repair defects in gastric tumor, which allows us to use PARP inhibitor to selectively induce apoptosis of gastric tumor cells. It has been shown that loss of CHFR prolongs the retention of PARP1 at DNA damage sites, which may suppress DNA damage repair and induce the accumulation of DNA lesions [13,18]. In particular, following DNA damage, massive protein PARylation occurs at DNA damage sites catalyzed mainly by PARP1 and the major substrate of protein PARylation is PARP1 itself, which is important for chromatin relaxation [13]. However, timely removal of PARP1 is equally important for the next step of DNA repair. Otherwise, PARP1 will occupy the sites of DNA damage and suppress DNA damage repair [13]. But lacking of CHFR is insufficient to induce cell apoptosis. Instead, it facilitates cell transformation and tumorigenesis [18]. However, when we further add PARP1 inhibitor treatment to completely trap PARP1 at DNA lesions, it may pass the threshold of cellular capacity to repair DNA lesions, thus cause tumor cells apoptosis (Figure 6). We have tested this strategy using Olaparib to treat gastric cancer cells and found that lacking CHFR sensitizes gastric cancer cells to Olaparib treatment. Thus, it provides a novel therapeutic approach for clinical gastric cancer treatment in future. Moreover, CHFR can be considered as the biomarker for PARP inhibitor treatment on gastric cancer in future. In addition, because lacking CHFR weakens DNA damage repair capacity in tumor cells, the cells are also hypersensitive to DNA damaging agents, such as MMS. Thus, it is also possible for treating gastric cells lacking the expression of CHFR with canonical DNA damaging agents that are often used in chemotherapy. In fact, cisplatin is the first line of chemo-drug for treating advanced gastric cancer. And our studies suggest that the expression of CHFR may serve as biomarker for predicting DNA damage repair capacity in gastric tumor cells.

In summary, our data show that CHFR gene expression is frequently silenced by DNA hypermethylation in gastric cancer. Gastric cancer cells lacking the expression of CHFR have defects in DNA damage repair and are hypersensitive to PARP inhibitors. Our studies suggest that DNA hypermethylation on CHFR may be a useful molecular marker to predict the efficacy of PARP inhibitors or other DNA damaging agents on gastric cancer.

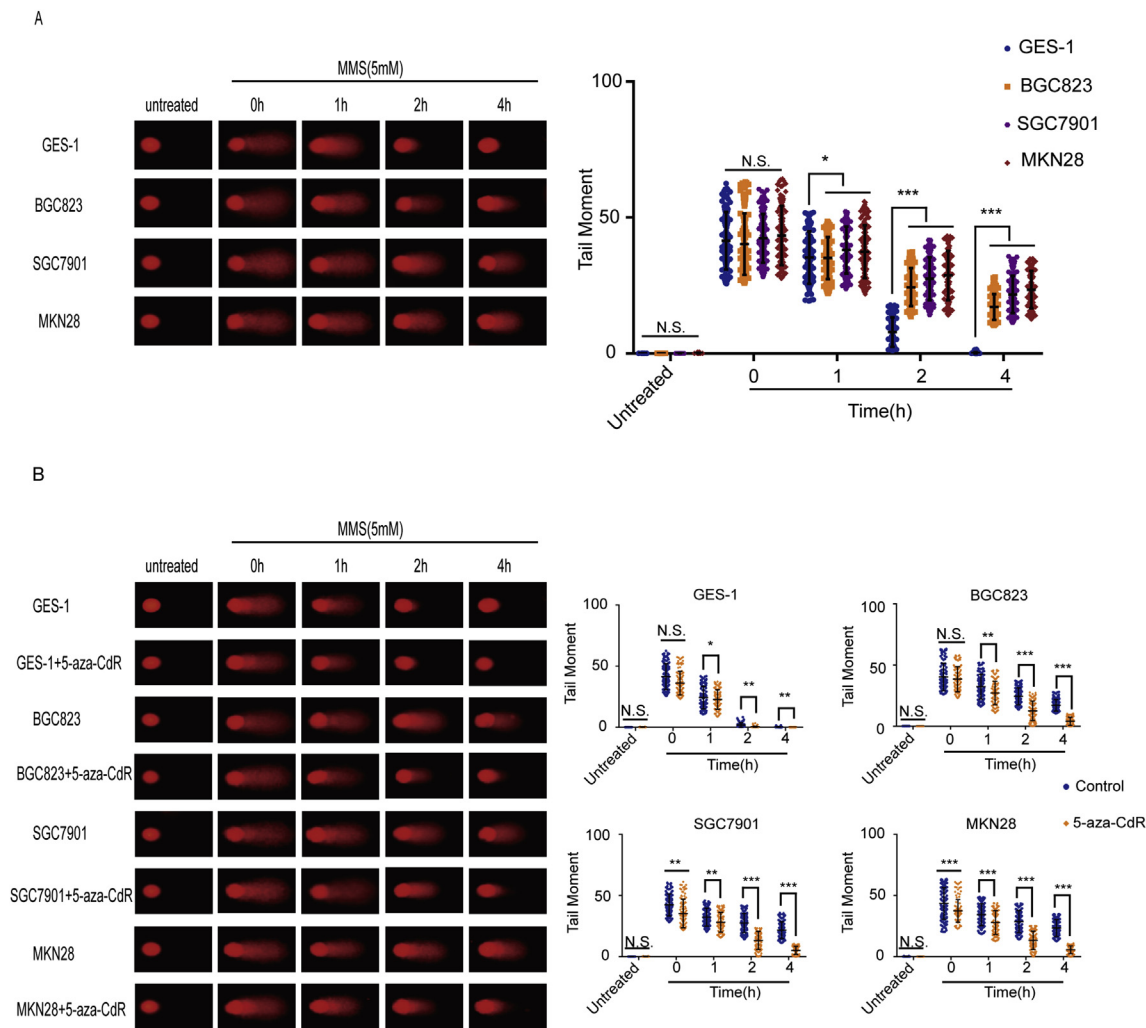
### **Materials and Methods**

#### *Cell Line and Tissue*

Three human gastric cancer cell lines (SGC7901, MKN28, and BGC823) and human normal gastric epithelium cell line (GES-1) were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). GES-1, MKN28, BGC823 were cultured in Dulbecco's Modified Eagle's Medium (GIBCO, Carlsbad, CA) and SGC7901 were cultured in RPMI Medium 1640 (GIBCO, Carlsbad, CA) supplemented with 10% fetal bovine serum



**Figure 3. DNA hypermethylation at *CHFR* promoter region is associated with DNMT1.** (A) 5-aza-CdR treatment restores the expression of CHFR. CHFR expressions were performed using RT-qPCR and Western blot. Cells were treated with different concentrations of 5-aza-CdR (5 μM and 10 μM) for 72 hours cDNA was prepared and qPCR was performed. The bars show levels of CHFR expression normalized to that of GAPDH. Cells were treated with 5-aza-CdR (10 μM) for 72 hours and lysed with NETN300. Western blot was performed with anti-CHFR antibody and GAPDH was used as a protein loading control. (B) 5-aza-CdR treatment inhibits the DNA methylation of the CpG islands at *CHFR* promoter region. Indicated cell lines were treated with 10 μM of 5-aza-CdR for 72 hours. The bisulfite PCR products were cloned into pMD-19T, and at least 10 clones from each cell line were sequenced. Open and closed areas represent unmethylated and methylated CpG dinucleotides, respectively. (C) DNMT1 is recruited to the promoter region of *CHFR*. ChIP assays on *CHFR* gene locus were performed using anti-DNMT1 antibody. An irrelevant IgG was used as a control shown as the dotted lines. qPCR amplification regions are indicated in the schematic diagram. The sequence of each primer is shown in Supplemental Table 2. \*:  $P < 0.05$ ; \*\*:  $P < 0.01$ ; \*\*\*:  $P < 0.001$ .



**Figure 4. Lacking CHFR causes DNA damage repair defects.** (A) DNA damage repair defects are examined with comet assays under alkaline condition. The gastric cancer cells were treated with MMS. Comet tail moments were examined to indicate the DNA damage repair kinetics. (B) Restoration of CHFR expression by 5-aza-CdR treatment rescues DNA damage repair. Cells were treated with 10  $\mu$ M 5-aza-CdR followed by MMS treatment. Data are represented as mean  $\pm$  S.D. as indicated from three independent experiments. \*:  $P < 0.05$ ; \*\*:  $P < 0.01$ ; \*\*\*:  $P < 0.001$ .

(GIBCO), 100  $\mu$ g/mL penicillin and 100  $\mu$ g/mL streptomycin, at 37  $^{\circ}$ C with 5%  $\text{CO}_2$  in a humidified incubator. Paired 26 primary gastric cancer specimens with 26 samples of adjacent stomach mucosa were collected from the Department of Surgery, Peking University Third Hospital, after acquisition of informed consent from each patient. The hospital institutional ethical review committee approved this study protocol.

#### DNA and RNA Extraction

Genomic DNA and total RNA were extracted using the PureLink<sup>®</sup> Genomic DNA Mini Kit (Invitrogen) and Trizol reagent (Invitrogen), respectively, according to the vendors' protocols. The quantity and quality of DNA and RNA were measured with a NanoDrop 2000 One C Spectrophotometer (Thermo Fisher Scientific). The integrity was further checked by electrophoresis in 1% agarose gel.

#### Sodium Bisulfite Conversion and Sample Preparation

Genomic DNA was bisulfite-modified using the EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA, USA) and the converted DNA was PCR-amplified using Taq<sup>™</sup> Hot Start Version

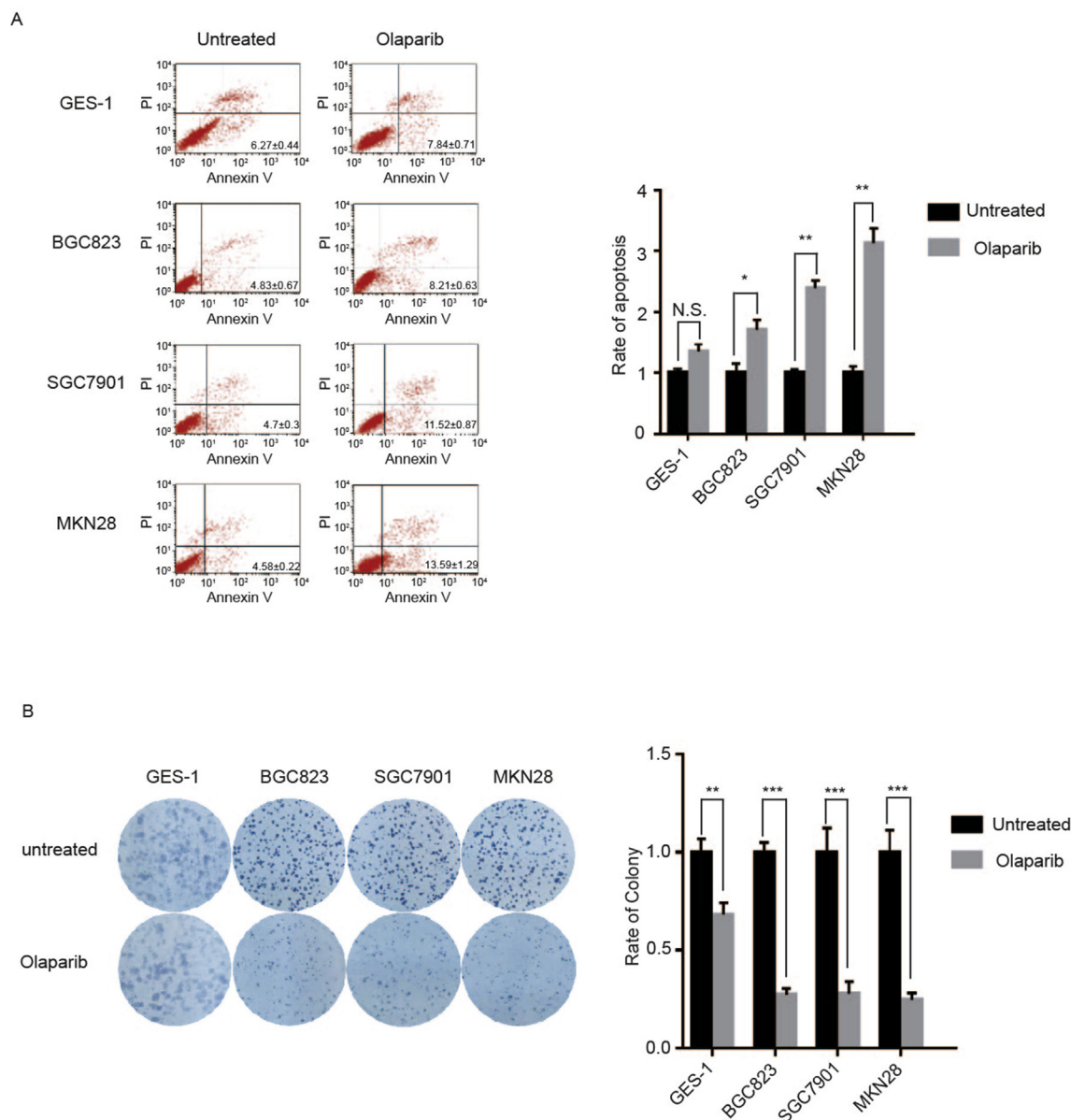
(TAKARA) in accordance with the provided guidelines. Primers used for PCR amplifications were forward 5'-TTTTTATTTTATAGGGAA TATTTTTTGG-3', reverse 5'-TACACACAATAAATCACACA AATCC-3'. The PCR products were electrophoresed in 1% agarose gels and purified using a TIANquick Midi purification kit (Tiangen Biotech) following the manufacturer's instructions. The purified products were then ligated into pMD-19T and transformed into DH5 $\alpha$  competent cell. The successful construction of cloning plasmids was identified by colony PCR and ten positive clones for each subject were randomly selected for sequencing.

#### Colony Formation Assay

Gastric adenocarcinoma cells were seeded at  $1 \times 10^3$  cells/well in 6-well plates and allowed to grow for 10 days with 1  $\mu$ M Olaparib treatment. The colonies were washed with PBS, fixed in methanol, and stained with Giemsa for colony visualization and counting.

#### Alkaline Comet Assay

Single-cell gel electrophoretic comet assays were performed under alkaline conditions. Briefly, cells were treated with or without 5 mM

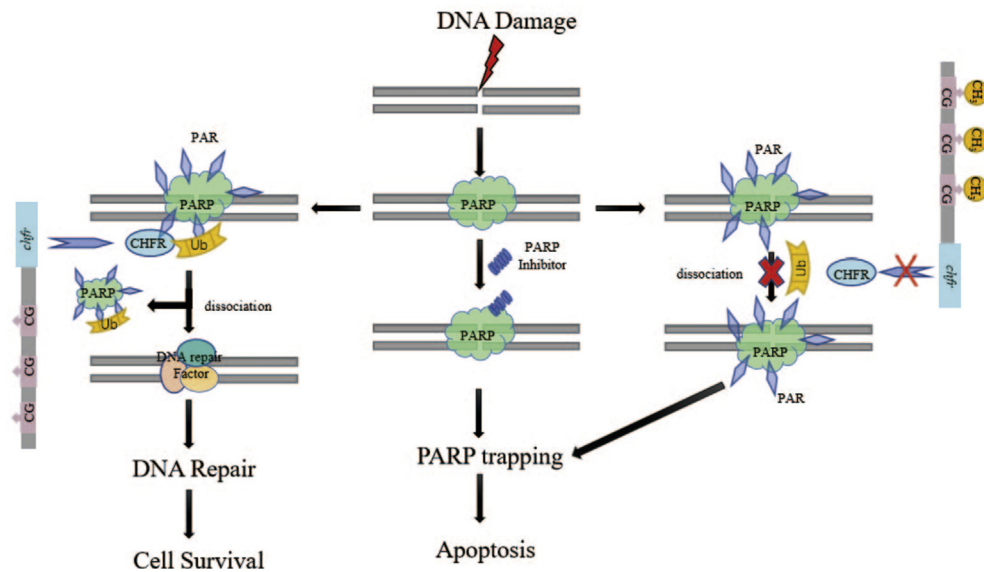


**Figure 5. Lacking CHFR sensitizes gastric cancer cells to PARP inhibitor treatment.** (A) Olaparib treatment induces gastric cancer cell apoptosis. Cells were treated with 5  $\mu$ M of Olaparib for 24 hours, and then analyzed by dual-parameter flow cytometry using Annexin V-FITC and PI double staining. Representative dot plot data from three independent experiments are shown in the left panel, and the histogram graph in the right panel represents the percentage of dual-parameter positive cells pooled from three independent experiments. (B) Gastric cancer cell sensitivity to PARP inhibitor is examined by colony formation assays. Representative images of colonies in plates stained with Giemsa. Images were acquired with a Nikon 90i with a DXM 1200C. Data are presented as the mean  $\pm$  SD of three independent experiments. N.S.: non-significant; \*:  $P < 0.05$ ; \*\*:  $P < 0.01$ ; \*\*\*:  $P < 0.001$ .

MMS and allowed to recover in normal culture medium for the indicated time at 37  $^{\circ}$ C. The cells were collected and rinsed twice with ice-cold PBS;  $2 \times 10^4$ /ml cells were combined with 1% LMP agarose at 42  $^{\circ}$ C at a ratio of 1:3 (v/v) and immediately pipetted onto slides. For cellular lysis, the slides were immersed in the alkaline lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM tris-Hcl, 1% Triton X-100, and 1% N-lauroylsarcosine sodium salt, pH > 10) overnight at 4  $^{\circ}$ C. Then, the slides were subjected to electrophoresis at 20 V for 25 min (0.6 V/cm) and stained in 10  $\mu$ g/ml propidium iodide for 30min. All images were taken with a fluorescence microscope and analyzed by the Comet Assay IV software program.

#### Cell Lysis and Western Blotting

Cells were lysed with NETN100 buffer containing 0.5% NP-40, 50 mM tris-HCl pH 8.0, 2 mM EDTA and 100 mM NaCl. Western blotting was performed following standard protocol as described previously [19]. To assess CHFR protein levels, 15  $\mu$ g of total protein from 70% to 80% confluent cell cultures were separated on 10% SDS-PAGE gels using the Criterion or Ready Gel systems (Bio-Rad Laboratories) and immunoblotted to PVDF membranes (Merck Millipore). Following 1 hour of incubation in a blocking solution of 5% nonfat dry milk and 0.1% TBS-Tween 20, a polyclonal antibody against CHFR (Proteintech, 12169-1-AP) was used at a 1:500 dilution in 0.5% nonfat dry milk and 0.05% TBS-Tween 20 and



**Figure 6.** A schematic diagram shows that loss of CHFR by DNA hypermethylation resulting in sensitizing gastric carcinoma cells to PARP inhibitor treatment.

incubated overnight at 4 °C. CHFR was detected by hybridization with a goat antimouse/horseradish peroxidase (HRP) secondary antibody (Cell Signaling Technology) at a 1:5000 dilution in TBST. For a loading control, the blots were immunoblotted with an antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control. The anti-GAPDH antibody (Abcam, ab8245) was used at a 1:1000 dilution and detected with a goat antimouse/HRP antibody at a 1:5000 dilution, both in 5% nonfat dry milk and 0.05% TBST. The SuperSignal West Pico PLUS chemiluminescent kit (Thermo) was used for detection.

### Immunohistochemistry

Paraffin embedded, formalin fixed biopsies from 26 patients were obtained and the malignant lesions were compared with normal tissue from the same patient from a distant site. Sections were dewaxed and then rehydrated and rinsed in phosphate buffered saline (PBS). After antigen retrieval in a microwave oven set at full power (720 W) for 15 min in pH 6.0 citrate buffer, the slides were washed in PBS and incubated with primary antibody for 1 hour at 37 °C. The CHFR protein expression was measured using the primary anti-CHFR polyclonal antibody at a dilution of 1:100. Followed the sections were incubated with the secondary antibody for 1 hour at 37 °C. Between each incubation step the slides were washed three times with PBS. A negative control was included in each experiment by omitting the primary antibody. Pictures were taken under the same illuminating conditions for each sample. The staining intensity of epithelial cells was measured in 20 microscopic fields belonging to the malignant portion of the sample and in 20 fields of the normal tissue, for each patient, with a Leica Quantimet Analysis System. For IHC using 5-mc antibody (Millipore, MABE146), after antigen retrieval, the slides were firstly immersed in 2 N HCl for 45 min at room temperature and washed in PBS. Sections were covered with 100  $\mu$ l anti-5-MeCyd monoclonal antibody (5  $\mu$ g/ml) and incubated for 1 hour at 37 °C, followed by incubated with the secondary antibody for 1 hour at 37 °C.

### Quantitative RT-PCR Assay

Total RNA was prepared using TRIzol (Invitrogen) and used for synthesis of first strand cDNA with Superscript II reverse transcriptase (Invitrogen). Quantitative PCR was performed using SYBR® Premix Ex Taq™ II (TAKARA, China) in CFX96™ Real-time systems (BIO-RAD). Primers for qPCR reactions are summarized in Supplemental Table S1. GAPDH mRNA was used for normalization. The mean value was calculated from three independent experiments.

### Detection of Apoptosis

Apoptotic cells were quantified by measuring externalized phosphatidylserine (PS) assessed by uptake of annexin V-FITC and propidium iodide (PI). After various experimental treatments, cells were stained with an annexin V-FITC apoptosis detection kit (Invitrogen, USA). Briefly, harvested cells were rinsed once with phosphate-buffered saline (PBS) and then resuspended in 200  $\mu$ l of 1 $\times$  binding buffer and 5  $\mu$ l annexin V-FITC, and then incubated at room temperature for 10 min in the dark. Washed cells in 200  $\mu$ l of 1 $\times$  binding buffer and resuspended in 190  $\mu$ l binding buffer and added 10  $\mu$ l PI. The population of apoptotic cells was analyzed immediately by flow cytometry (FACSCalibur, BD Bioscience).

### Chromatin Immunoprecipitation

Briefly, cells were harvested and their proteins were cross-linked to DNA by incubation in 1.0% formaldehyde for 10 min at 37 °C. The formaldehyde-fixed cells were subjected to settle on ice for 10 min and then spun down by brief centrifugation, after which the supernatant was carefully aspirated. The cells were then washed with ice-cold PBS-containing protease inhibitors and resuspended in lysis buffer [1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.0), and protease inhibitor]. The nucleoprotein complexes were sonicated to reduce the sizes of the DNA fragments to 200–1000 bp and immunoprecipitated for overnight at 4 °C with rotation using anti-DNMT1 antibody (GeneTex, GTX30364). The resultant immune complexes were collected



using protein G-agarose beads, after which the DNA was purified by phenol-chloroform extraction, precipitated with ethanol, and resuspended in distilled water. About 1:100 of the precipitated DNA was used for PCR, and 1:100 of the solution before adding antibody was used as an internal control for the quantitative accuracy of the DNA. Primers for ChIP quantitative PCR are summarized in [Supplemental Table S2](#). The mean value was calculated by three independent experiments.

### Acknowledgments

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### Conflict of Interest

None declared.

### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.tranon.2019.10.004>.

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