Inhibition of Serine Metabolism Promotes Resistance to Cisplatin in Gastric Cancer

Xiaoya Zhao1,2,* Jianfei Fu2,* Wanfen Tang3 Liangliang Yu4 Wenzia Xu1

1. Central Laboratory, Jinhua Hospital of Zhejiang University, Jinhua 321000, Zhejiang Province, People’s Republic of China; 2. Department of Medical Oncology, Sir Run Run Shaw Hospital, Medical School of Zhejiang University, Hangzhou 310000, Zhejiang Province, People’s Republic of China; 3. Department of Medical Oncology, Jinhua Hospital of Zhejiang University, Jinhua 321000, Zhejiang Province, People’s Republic of China; 4. Department of Gastroenterology, Sir Run Run Shaw Hospital, Medical School of Zhejiang University, Hangzhou 310000, Zhejiang Province, People’s Republic of China.

*These authors contributed equally to this work.

Correspondence: Liangliang Yu
Department of Gastroenterology, Sir Run Run Shaw Hospital, Medical School of Zhejiang University, No. 3 Qingchun Road, Jianggan District, Hangzhou 310000, Zhejiang Province, People’s Republic of China.
Tel +86 571-86006646
Email ydy30838@163.com

Wenzia Xu
Central Laboratory, Jinhua Hospital of Zhejiang University, No. 351 Mingyue Street, Wucheng District, Jinhua 321000, Zhejiang Province, People’s Republic of China.
Tel +86 579 82553851
Email xuwenxia@zju.edu.cn

Introduction

Gastric cancer is one of the most common malignant tumors, with the second-highest incidence and third highest mortality in China.1 Patients with gastric cancer show “three high and three low characteristics”, including high incidence, metastasis, and mortality rates, as well as low early diagnosis, radical cure, and 5-year survival rates.2 At present, gastric cancer is mainly treated with surgery, radiotherapy, and chemotherapy. However, the mechanism of occurrence and development of gastric cancer remains unclear. The high rates of metastasis and recurrence, along with drug tolerance have become the main obstacles to improve the long-term survival rate of patients.3,4 Therefore, the identification of new molecular prognostic markers...
and therapeutic targets is necessary to improve the clinical prognosis of patients with gastric cancer.

Cisplatin is one of the most commonly used chemotherapeutic agents for the treatment of numerous types of cancer (eg, gastric, non-small-cell lung, bladder, testicular, ovarian, head and neck, and other malignancies). However, the use of cisplatin in clinical treatment is limited by two major problems. Cisplatin exerts many toxic and side effects (eg, kidney damage, deafness, peripheral neuropathy, etc.), leading to a decrease in the overall efficacy of the drug. Furthermore, tumors that initially respond to treatment with cisplatin often develop resistance to further treatment with platinum after tumor recurrence. The overall mechanisms of resistance to platinum include increased DNA repair, altered drug accumulation in the cell, and increased intracytoplasmic inactivation of drugs.

Metabolic reprogramming is one of the 10 characteristics of tumors, among which amino acid metabolic reprogramming has attracted increasing attention. In addition to glutamine metabolism, serine/glycine metabolism is also necessary for tumor cells. Serine is a non-essential amino acid in the human body; however, it is an essential amino acid in specific circumstances for tumors. It is obtained from the external environment through transporters and also synthesized by the serine synthesis pathway, as necessary. In 1955, the endogenous de novo synthesis pathway of serine was first observed in tumor cells. Phosphoglycerate dehydrogenase (PHGDH) converts approximately 10% of the 3-phosphoglyceric acid produced during glycolysis into 3-phosphatedehydropyravate, a precursor of serine, and is subsequently catalyzed by phosphoserine aminotransferase 1 and phosphoserine phosphatase to produce serine. The reduction of exogenous serine concentration or targeted inhibition of PHGDH can effectively play an anti-tumor role.

However, the effects of reducing the exogenous serine concentration or inhibiting PHGDH combined with chemotherapy remain unclear.

In this study, we discovered the mechanism of inhibiting serine metabolism leads to cisplatin resistance in gastric cancer. Which laid a foundation for further investigation of the relation between nutrient metabolism and tumor chemotherapy.

**Materials and Methods**

**Reagents**

Human gastric cancer cell lines SGC7901, BGC823, and MGC803 were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). RPMI-1640 medium (31800–105) was obtained from Gibco (Grand Island, New York, USA). RPMI-1640 w/o Amino, sodium phosphate (powder) was purchased from US Biological (Salem, MA, USA). Fetal bovine serum (11011–8611) was obtained from Every Green (Hangzhou, China). Penicillin-streptomycin solution (GNM15140) and trypsin (GNM25200) were purchased from Genome (Hangzhou, Zhejiang, China). Arginine, asparagine, aspartic acid, cysteine dihydrochloride, glutamic acid, glycine, histidine, proline, tyrosine, valine, glutamine were obtained from Sigma (Louis, MO, USA). Hoechst 33258 (C1017), horseradish peroxidase-conjugated goat anti-Mouse, and anti-Rabbit IgG were purchased from Beyotime Biotechnology (Shanghai, China). Micrococcal nuclease (MNase; 2910A) was obtained from Takara (Osaka, Japan). Radioimmunoprecipitation assay lysis buffer (FD009), BCA Protein Assay Kit (FD2001), and enhanced chemiluminescence kit (FD800) were purchased from Fdbio Science (Hangzhou, Zhejiang, China). CBR-5884 (HY-100012) and NCT-503 (HY-101966) were purchased from MedChemExpression (Shanghai, China). JIB-04 (S7281) was obtained from Sigma (Louis, MO, USA). Rabbit anti-poly-ADP ribose polymerase (anti-PARP; 13371-1-AP) and rabbit anti-Caspase3 (66470-2-Ig) were purchased from Proteintech (IL, USA). Rabbit anti-Tubulin (AF0001) and mouse anti-Actin (AA128) were obtained from Beyotime Biotechnology. Rabbit anti-γH2AX (ab81299) was purchased from Abcam (Cambridge, UK); rabbit anti-H3 (#4499) and rabbit anti-H3K4 me3 (#9751) were purchased from Cell Signaling Technology (Danvers, MA, USA). TR1zol (15596026) was purchased from Ambion (Carlsbad CA, USA) and dimethyl sulfoxide (D2650) was obtained from Sigma.

**Cell Culture**

Human gastric cancer cell lines SGC7901, BGC823, and MGC803 were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. All cells were incubated at 37°C in a 5% CO₂ humidified incubator. Cells were grown to 70–90% confluence.

**Preparation of Different Concentrations of Serine**

RPMI-1640 w/o Amino, Sodium Phosphate (powder), sodium bicarbonate, and 19 types of amino acids (excluding serine), were dissolved in double-distilled water to generate...
serine-free 1640 medium, termed 0×Ser. We defined the concentration of serine in the 1640 complete medium (30 μg/mL) as 1×Ser. The 100×Ser (3,000 μg/mL) solution was prepared in advance, and subsequently added to serine-free 1640 medium to produce 1/4×Ser (7.5 μg/mL), 1×Ser (30 μg/mL), 4×Ser (120 μg/mL), and 8×Ser (240 μg/mL), which were used in the following experiments.

Cell Viability

Cells were plated onto 96-well plates at a density of 1×10^4 cells per well, and cultured in 100 μL complete medium for 24 h. Subsequently, a complete medium was replaced with medium containing 0.6 μg/mL, 0.8 μg/mL, and 3 μg/mL cisplatin under different concentrations of serine or the inhibitor of PHGDH for 24 h. This was followed by the addition of 100 μL medium containing 10 μL Cell Counting Kit-8 (Beyotime Biotechnology) agent to each well for 1 h after discarding the supernatant. The optical density values at 450 nm were determined using a Microplate Reader (Synergy HT; Bio-Tek Instruments, USA).

Western Blotting Analysis

Briefly, the whole-cell protein was obtained using radioimmunoprecipitation assay lysis buffer (RIPA) containing 1 mM phenylmethylsulfonyl fluoride. Equal loading of lysate protein was determined by standardization using the BCA Protein Assay Kit and a Microplate Reader (Synergy HT; Bio-Tek Instruments). Proteins were separated through electrophoresis using sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and transferred to polyvinylidene difluoride membranes (BioRad, Hercules, California, USA). The membranes were blocked at room temperature for 1 h in tris-buffered saline containing 5% fat-free powdered milk and incubated overnight with primary antibodies at 4°C. Subsequently, the membranes were incubated with secondary antibody for 2 h at room temperature after washing with tris-buffered saline containing 0.1% Tween 20. Proteins were detected using the FDbio-Pico enhanced chemiluminescence kit and quantified with the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Hoechst 33258 Staining

Cells were plated onto 24-well plates at a density of 1×10^5 cells per well, and cultured in 1,000 μL complete medium for 24 h. Cells were cultured for 24 h after replacing the supernatant with the medium including 0.6 μg/mL cisplatin and extra serine. The cells were fixed with methanol for 10 min and washed thrice with phosphate-buffered saline. Three hundred μL of Hoechst 33258 staining solution was added for 3–5 min at room temperature. The staining solution was subsequently removed and the cells were washed thrice with phosphate-buffered saline, each time for 3–5 min. Cells were observed under a fluorescence microscope and quantified with the ImageJ software (National Institutes of Health).

MNsase Sensitivity Assay

SGC7901 cells were treated with NCT-503, JIB-04 and cisplatin for 24 h. Cells were treated with lysis buffer (10 mM Tris-hydrochloride [pH 8.0], 10 Mm magnesium dichloride, 1 mM dithiothreitol and 0.5% NP-40), incubated on ice for 15 min, centrifuged at 13,000 g for 4 min at 4°C. The supernatant was removed, nuclei were pelleted and digested with Mnase at a concentration of 4 U/200 μL digestion buffer (15 mM Tris-hydrochloride [pH 7.4], 60 mM potassium chloride, 15 mM sodium chloride, 0.25 M sucrose, 1Mm calcium chloride and 0.5 mM dithiothreitol) for 10 min at 37°C. Genomic DNA was extracted using a genomic DNA mini preparation kit (Beyotime Biotechnology), purified and separated through electrophoresis using 1.5% agarose gels.

Statistical Analysis

Data are presented as the mean ± standard deviation. Statistical analyses were performed with the unpaired, two-tailed Student’s t-test using GraphPad Prism 6.02 for Mac (GraphPad Prism Software Inc., San Diego, CA, USA). A p<0.05 denoted a statistically significant difference.

Results

Serine Concentration Was Positively Correlated with the Sensitivity of Gastric Cancer Cells to Cisplatin

We examined the activity of gastric cancer cells treated with cisplatin under different concentrations of serine to investigate the effect of serine concentration on sensitivity to this drug. The concentration of serine in the 1640 complete medium was 30 μg/mL, which was defined as 1×Ser. We treated gastric cancer cells SGC7901, BGC823 and MGC803 with 0×Ser, 1/4×Ser, 1×Ser, 4×Ser and 8×Ser. The lower (0×Ser, 1/4×Ser) and higher (4×Ser, 8×Ser) concentrations of serine had no effect on cell toxicity and apoptosis (Figures 1A–E and S1A). Cisplatin had a significantly higher toxic effect on gastric cancer cells in the 4×Ser and 8×Ser groups than the 1×Ser group (Figure 1A–C). However, the toxicity of cisplatin in the 0×Ser and 1/4×Ser groups was significantly lower than that observed in the 1×Ser group (Figure 1A–C). Typical
nucleus condensation, as the morphological marker of apoptosis, was observed using Hoechst 33258 staining. The experiment showed that higher concentrations of serine (4×Ser, 8×Ser) significantly promoted the apoptosis of cells induced by cisplatin (Figures 1D and E). These results indicate that the concentration of serine was positively related to the sensitivity of gastric cancer cells to cisplatin.

**Inhibition of PHGDH Leads to Resistance to Cisplatin in Gastric Cancer Cells**

Serine can be obtained from the external environment, and also synthesized through the serine synthesis pathway from the glycolysis flux (Figure 2A). PHGDH is highly expressed in numerous types of human tumors and positively correlated with the progression of breast cancer, colorectal cancer and poor prognosis. We found that high PHGDH expression was significantly associated with decreased overall survival, progression-free survival and post-progression survival in patients with gastric cancer (Figures 2B, S1B, and S1C). We inhibited PHGDH by NCT-503 or CBR-5884 (the specific inhibitors of PHGDH) and found that the proliferation of BGC823 cells was suppressed (Figure S1G), while the apoptosis was not observed (Figures 2E, F, S1I and S1J). Moreover, the knockdown of PHGDH cannot induce apoptosis of cells (Figure S1H). Then, we treated gastric cancer cells with NCT-503 or CBR-5884 in combination with platinum-based drugs (cisplatin and oxaliplatin), to investigate the relationship between PHGDH and the sensitivity to these agents. Cisplatin or oxaliplatin combined with PHGDH inhibitors led to a significant increase in cell survival rate versus monotherapy with cisplatin or oxaliplatin (Figures 2C, D and S1D–F). Moreover, the expression levels of cleaved PARP and Caspase3 (two apoptotic marker proteins) were significantly reduced in the combined treatment group versus the monotherapy group (Figures 2E, F, S1I and S1J). We found lower survival and higher apoptosis rates in cells treated with NCT-503, cisplatin and extra serine relative to those recorded in cells treated with cisplatin and NCT-503 (Figure 2G and H). These results suggest that inhibition of PHGDH induced resistance to cisplatin in gastric cancer cells, whereas supplementation with serine restored the sensitivity of gastric cancer cells to this chemotherapeutic agent.
Inhibition of PHGDH Decreases Cisplatin-Induced DNA Damage in Gastric Cancer Cells

Cisplatin kills cancer cells mainly by destroying DNA in the form of Pt-d (GpG), leading to DNA double-strand breaks.\(^{16,17}\) It has been reported that the levels of $\gamma$H2AX are positively correlated with the severity of DNA damage.\(^{18}\) We treated gastric cancer cells with cisplatin for 2 h, and replaced the medium with fresh complete medium with or without NCT-503 and CBR5884 for 0, 4, 8, 12 and 24 h to examine the cisplatin-induced DNA damage in gastric cancer cells following the inhibition of PHGDH. The levels of $\gamma$H2AX protein in the NCT-503 and CBR5884 treatment groups were significantly lower than those reported in the control group at all time points (Figure 3A–C). The results of the gray-scale quantitative analysis showed that the growth rate of $\gamma$H2AX was...
significantly lower than that observed in the control group (Figure 3D–F). These results illustrate that the inhibition of PHGDH reduced the DNA damage induced by cisplatin in gastric cancer cells.

**Inhibition of Serine Metabolism Leads to Histone Demethylation**

We also examined the methylation of H3K4 to obtain a deep insight into the blockage of DNA damage on account of inhibition of serine metabolism. The results of our previous study showed lower H3K4 tri-methylation in drug-resistant gastric cancer cells. Moreover, decreased H3K4 tri-methylation was observed after serine deprivation or inhibition of PHGDH (Figure 4A). JIB-04 is a broad-spectrum selective inhibitor of Jumonji histone demethylase. We treated cells with cisplatin and NCT-503 combined with JIB-04. This treatment significantly decreased cell activity versus cisplatin co-administered with NCT-503 only (Figure 4B). The levels of H3K4 tri-methylation were promoted by treatment with JIB-04 (Figure 4C). Furthermore, we examined the DNA damage in cells treated with JIB-04 to extend these findings. Strikingly, the expression of γH2AX was markedly increased (Figure 4D and E). Studies have shown that low coagulation DNA encased in chromatin is more severely damaged than tightly packed chromatin under ionizing radiation, while the spatial structure of chromatin is regulated by histone post-translational modifications (eg, acetylation and methylation). Based on this evidence, we used an indirect method of MNase digestion to analyze chromatin condensation and investigate the mechanism through which NCT-503 reduces the cisplatin-induced DNA damage in gastric cancer cells. We found that the abundance of mononucleosomes and binucleosomes in the cisplatin and NCT-503 combination treatment group was lower than that noted in the cisplatin monotherapy group (Figure 4F). In contrast, the abundance of mononucleosomes and binucleosomes could be significantly increased by treatment with JIB-04 combined with cisplatin and NCT-503 (Figure 4F). Thus, a low concentration of serine enhances chromatin compactness by inhibiting H3K4 tri-methylation, resulting in decreased cisplatin-induced DNA damage.

**Discussion**

Tumors require a variety of nutrients for the synthesis of biological macromolecules to maintain their growth and proliferation. Serine provides the necessary precursor for the synthesis of proteins, nucleic acids, and lipids essential for the growth of cancer cells. The association between cancer and serine biosynthesis was first demonstrated in rat liver tissue. We observed the activity and apoptosis of cells induced by cisplatin under different concentrations of serine to investigate the link between the sensitivity of gastric cancer cells to cisplatin or oxaliplatin and serine metabolism. Unexpectedly, lower serine concentration alleviated the sensitivity of gastric cancer cells to cisplatin or oxaliplatin. Serine can be obtained through the de novo serine synthesis pathway catalyzed by PHGDH, a rate-limiting enzyme that initiates this pathway. Numerous studies have linked the expression levels of PHGDH with the activity of the serine synthesis pathway. Thus far, PHGDH has been found to be highly expressed in...
various types of cancer (e.g., breast,\textsuperscript{29} bladder,\textsuperscript{30} glioma,\textsuperscript{31} cervical,\textsuperscript{32} melanoma,\textsuperscript{33} non-small-cell lung,\textsuperscript{34} colorectal\textsuperscript{11} and gastric\textsuperscript{35}), dysregulating serine metabolism and contributing to tumor progression. Inhibition of PHGDH activity by siRNA or inhibitors can disrupt the serine synthesis pathway and restrain the growth and proliferation of tumor cells.\textsuperscript{9,36}

Recent studies have shown that the combination of PHGDH inhibitors and chemotherapeutic drugs in breast cancer,\textsuperscript{13} multiple myeloma,\textsuperscript{37} melanoma,\textsuperscript{38} lung adenocarcinoma,\textsuperscript{39} renal cell carcinoma\textsuperscript{40} and hepatocellular carcinoma\textsuperscript{41} can increase the sensitivity of cells to chemotherapy. It is meaningful to clarify the mechanism of the combination of PHGDH inhibitors with chemotherapy in different types of tumors for the development of targeted drugs. However, thus far, studies have not investigated this mechanism in gastric cancer cells. In this study, we found that the combination of cisplatin with PHGDH inhibitors decreased the efficacy of cisplatin in gastric cancer. Notably, the addition of extra serine reversed the resistance of gastric cancer cells to cisplatin. The mechanism may involve the suppression of serine synthesis by inhibiting the metabolic enzyme activity of PHGDH, reducing the concentration of serine in cells. The relationship between resistance to cisplatin and serine metabolism in vivo, and whether controlling the concentration of serine can guide chemotherapy in clinical practice remains to be investigated.

The continuous use of cisplatin will activate the protective signaling regulatory network resulting in the development of drug resistance.\textsuperscript{42,43} It has been reported that the mechanism of DNA damage and repair can be affected by cell metabolism through three main links.\textsuperscript{44} First, covalent modifications of DNA and histones are regulated by different metabolic pathways (e.g., methylation, hydroxylation, acetylation, O-GlcNAcylation and hydroxylation), which affect the folding and remodeling of chromatin.\textsuperscript{45,46} Second, glutamine, aspartate, serine and other nutrients are necessary for the de novo synthesis of nucleotides as well as the content of nucleotides affects the repair and replication of DNA. Thirdly, the regulation of reactive oxygen species through different metabolic pathways can increase the oxidative damage to DNA, thus increasing the load of the DNA repair mechanism. Although tumor metabolism has attracted considerable attention in the past decade, the link between metabolism and DNA
damage, as well as repair in cancer remains to be explored. Therefore, we examined the degree of DNA damage in gastric cancer cells after treatment with PHGDH inhibitor, discovering that inhibition of PHGDH reduced DNA damage caused by cisplatin in gastric cancer cells.

Serine can be catalyzed to glycine by serine hydroxymethyltransferase. Subsequently, the dragged hydroxyl group is added to tetrahydrofolate to form N\textsuperscript{5}, N\textsuperscript{10}-methyl-tetrahydrofolate, which is incorporated into the folate cycle to provide a carbon unit for nucleotide synthesis and biological methyl groups, such as histone and DNA methyltransferase coenzyme, S-adenosine methionine.\textsuperscript{47} Histone methylation mainly occurs at sites such as H3K4, H3K9, H3K27, H3K36, H3K79 and H4K20.\textsuperscript{24,25,46} Previous studies provided evidence that the consequence of DNA damage and genome instability because of the demethylation of tri- or di-methylation of H3K9 caused by overexpression of lysine demethylase 4B (KDM4B) facilitated the failure of cytotoxic anti-cancer treatment.\textsuperscript{49,50} Furthermore, the depletion of PHD finger protein 2 (PHF2) increased the global levels of K3K9 me3 in progenitor cells, which could delay or impair DNA repair.\textsuperscript{51} Pippa et al demonstrated that following DNA damage, local H3K4 demethylation induced by lysine demethylase 5B (KDM5B) is vital for the recruitment of Ku70/80 and BRCT-Cancer susceptibility gene 1 (BRCA1) in DNA repair.\textsuperscript{52} In addition, lower expression of H3K4 tri-methylation was found in drug-resistant gastric cancer cells.\textsuperscript{19} We found that the levels of H3K4 tri-methylation were decreased following the removal of exogenous serine and endogenous serine synthesis was blocked by treatment with NCT-503. We used JIB-04 to improve the methylation modification state of H3K4 and found that the sensitivity of cells to cisplatin was reversed and DNA damage was increased. These results indicate that a low concentration of serine disrupted the damage and repair mechanism of DNA by reducing H3K4 tri-methylation, which promotes the tolerance of gastric cancer cells to cisplatin.

The spatial structure of chromatin plays an important role in regulating the response to DNA damage. It is mainly regulated by post-translational modifications, such as acetylation and methylation on histone lysine residues.\textsuperscript{26,53} Demethylated H3K4 can adjust the spatial structure of chromatin to fully expose the damaged site after the occurrence of DNA damage, thus facilitating the accurate repair by DNA repair molecules.\textsuperscript{19} Condensation of chromatin structure is mainly controlled by inter-nucleosomal and/or linker DNA–core interaction.\textsuperscript{54} We used the indirect method of MNase digestion to investigate the mechanism through which the expression of H3K4 tri-methylation regulates the structure of chromatin after treatment with NCT-503. We found that NCT-503 enhanced the spatial density of chromatin, while JIB-04 relieved the condensation of chromatin caused by NCT-503. According to the above results, we hypothesized that low concentrations of serine promote resistance of gastric cancer cells to cisplatin by decreasing H3K4 tri-methylation and strengthening the spatial density of chromatin to avoid the exposure of DNA to chemotherapeutic drugs. Further detailed investigation of the regulatory mechanism of H3K4 tri-methylation for the condensation of chromatin and the concentration of cisplatin binding to DNA will be performed in future studies.

**Conclusion**

The results of the present study revealed that the demethylation of H3K4, generated by inhibition of serine metabolism, prevented DNA damage and gave rise to resistance to cisplatin in gastric cancer cells. These findings provide an opportunity to combine serine supplement with chemotherapy for the treatment of patients with gastric cancer in the clinical setting.

**Abbreviations**

MNase, micrococcal nuclease; PHGDH, phosphoglycerate dehydrogenase; PSAT1, phosphoserine aminotransferase 1; PHF2, PHD finger protein 2; KDM4B, lysine demethylase 4B; KDM5B, lysine demethylase 5B; BRCA1, Breast-Cancer susceptibility gene 1.

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**Disclosure**

The authors report no conflicts of interest in this work.

**References**


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