Original Article
eIF5A2 regulates the resistance of gastric cancer cells to cisplatin via induction of EMT

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Abstract: Cisplatin is the first-line chemotherapy drug for gastric cancer (GC), but treatment failure often occurs due to development of resistance. The mechanism of cisplatin resistance remains a mystery. Eukaryotic translation initiation factor 5A2 (eIF5A2) is an important tumor-promoting factor and has been rarely studied in GC. This study aimed to investigate the role of eIF5A2 in cisplatin resistance of GC cells and its relationship with epithelial-mesenchymal transition (EMT). We found that it is negative correlation between cisplatin resistance and eIF5A2's expression in GC cells. Silencing of eIF5A2 enhanced the sensitivity of GC cells to cisplatin, while overexpression of eIF5A2 decreased sensitivity. Cisplatin treatment induced gene expression changes consistent with EMT. EMT was blocked and the sensitivity of GC cells to cisplatin was increased by inhibiting the expression of Twist, indicating that EMT regulates the sensitivity of GC cells to cisplatin. Knockdown of eIF5A2 was associated with upregulation of the epithelial markers E-cadherin and β-catenin, while the expression of mesenchymal markers vimentin and N-cadherin decreased, indicating that eIF5A2 can reverse the EMT process and block the effect of cisplatin on EMT-related markers. Knockdown or overexpression of eIF5A2 did not affect the sensitivity of gastric cancer cells to cisplatin by Twist siRNA. Altogether, these data suggest that eIF5A2 regulates the resistance of gastric cancer cells to cisplatin by mediating EMT, and support the conclusion that eIF5A2 may be a molecular target for anti-tumor therapy.

Keywords: eIF5A2, EMT, gastric neoplasms, cisplatin, drug resistance

Introduction

Gastric cancer (GC) is a type of high-incidence and high-fatality tumor, with more than 70% of new cases of GC occurring in developing countries [1]. Most patients have progressed to advanced GC at their first presentation, which seriously threatens their prospects for successful treatment. Chemotherapy is the main treatment for GC patients and cisplatin is the first-line drug, which exerts cytotoxicity through damage to DNA structure (i.e., cross-linking of nucleotides), leading to apoptosis of tumor cells. Unfortunately, chemotherapy resistance emerges readily, leading to tumor recurrence

[2]. Therefore, it is important to find an effective target at the level of specific genes and proteins in order to overcome cisplatin chemotherapy resistance.

Amplification of 3q26 is one of the most frequent chromosomal aberrations in many human cancers including lung [3], ovarian [4], esophageal [5], and prostate [6] carcinomas, suggesting the presence of candidate oncogenes in this region. Studies have identified an oncogene from this region, eukaryotic initiation factor 5A2 (eIF5A2), which plays an important role in regulating cell proliferation and apoptosis [7, 8]. Further functional studies have confirmed that

eIF5A2 is associated with the metastasis and progression of ovarian [9], hepatocellular [10], colorectal [11], and nasopharyngeal [12] carcinomas. Additionally, eIF5A2 overexpression can initiate tumor formation, promote cancer cell growth, and induce chemotherapy resistance. For example, eIF5A2 overexpression induced chemotherapy resistance of nasopharyngeal carcinoma cells to 5-fluorouracil [12, 13], while inhibiting the expression of eIF5A2 significantly enhanced the cytotoxic effect of cisplatin on oral squamous cell carcinoma (OSCC) [14]. In addition, 75 out of 160 GC tissue samples were found to overexpress eIF5A2, while only 7 tissue samples in matched non-tumor mucosal tissues showed overexpression (*P* < 0.001), suggesting that eIF5A2 may be a potential therapeutic target in GC [15]. However, there is still little research on the role of eIF5A2 and its correlation with cisplatin resistance in GC.

Epithelial-mesenchymal transition (EMT) is initiated and regulated by a variety of different cytokines and growth factors during tumor development. It is a complex and reversible process that leads to the loss of epithelial markers (such as E-cadherin and β-catenin) and the upregulation of mesenchymal markers (such as N-cadherin and vimentin), which can enhance the ability of cancer cells to migrate and invade [16]. Additionally, it has been confirmed that overexpression of eIF5A2 in Colorectal Carcinoma can activate EMT via c-myc-mediated regulation of MTA1 [17], and eIF5A2 was found to induce EMT in hepatocellular carcinoma by activating RhoA/Rac1 [10]. It has also been shown that tumor cells from gastric [18, 19], lung [20, 21], and breast [22] cancers can obtain multidrug resistance during the course of EMT. There are also studies showing that cisplatin can promote EMT in esophageal squamous cell carcinoma (ESCC) cells, and inhibition of EMT can enhance the chemosensitivity of ESCC cells to cisplatin. Therefore, taking into account the carcinogenic potential of EMT, it is important to study the possible role of EMT in drug resistance to common GC chemotherapy drugs such as cisplatin.

In the current study, we used undifferentiated HGC-27 cells, poorly differentiated BGC-823 and AGS cells, and well-differentiated MGC-803 cells to study the relationship between eIF5A2 and EMT, and the role of eIF5A2 in the resistance of GC cells to cisplatin. We con-firmed that the expression of eIF5A2 protein was negatively correlated with sensitivity to cisplatin. EMT occurred after treatment with cisplatin, while knockdown of Twist or eIF5A2 blocked EMT and enhanced the sensitivity of GC cells to cisplatin. Interestingly, silencing or overexpression of eIF5A2 does not affect the sensitivity of gastric cancer cells to cisplatin after inhibition of EMT. Taken together, our data suggest that eIF5A2 regulates the resistance of gastric cancer cells to cisplatin via induction of EMT.

Materials and methods

Cell culture and reagents

The GC cell lines MGC-803, BGC-823, HGC-27, and AGS were cultured in RPMI-1640 medium (Gibco, Massachusetts, USA) with 10% fetal bovine serum (FBS; Gibco, Massachusetts, USA). The cells were cultured at 37°C with 5% CO_2. The cells were passaged with 0.25% trypsin digestion. Cisplatin was obtained from Sigma-Aldrich Company and stock solutions were prepared in dimethyl sulfoxide (DMSO).

CCK-8 cell viability assay

Cell viability was analyzed with the Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) according to the manufacturer's protocols. GC cells (3 × 10^3 cells/well) were seeded into 96-well culture plates and allowed to attach for 12 h. The culture medium was then replaced with complete medium containing cisplatin at indicated doses for 48 h. Finally, CCK-8 solution (10 μl/well) was added and the cells were incubated at 37°C for 2 h, and then absorbance was assessed at 450 nm using a MRX II microplate reader (Dynex, Chantilly, VA, USA). The cell viability was calculated as a percentage of untreated control cells. Each experiment was performed and repeated three times.

EdU incorporation assay

Cell proliferation was assessed by Click-iT EdU Imaging Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Cells were treated with cisplatin (0, 3.125, 6.25, 12.5, 25, 50 μmol/L) or both cisplatin and Twist siRNA for 48 h. Then incubated with 10 μmol/L EdU for 2 h at 37°C. The cells were fixed in 3.7% formaldehyde for 30 min at room temperature, washed twice with PBS. Then permeabilized in 0.5% Triton X-100 for 20 min at room

temperature, washed twice with PBS. Incubated with 100 µL Click-iT® reaction cocktail for 30 min in the dark, followed by 1 mL of 1X Hoechst 33342 (1:2000) for 30 min. Three random fields of view per slide were captured using a fluorescence microscope (Olympus, Tokyo, Japan) and the numbers of proliferating cells (EdU-positive) were counted. Each experiment was performed and repeated three times.

Western blot analysis

GC cells were collected and whole cellular extracts were lysed in RIPA buffer (Cell Signaling Technology (CST), Danvers, MA, USA) containing protease inhibitors according to the manufacturer's instructions. Protein concentration was quantified using the BCA Protein kit (Thermo Fisher Scientific, Rockford, IL, USA). Equal amounts of proteins were separated by 10% and 15% SDS-PAGE and electrophoretically transferred to PVDF membranes (Millipore, Billerica, MA, USA), and then blocked with TBS/T containing 5% bovine serum albumin (BSA) for 1 h at room temperature. Membranes were then incubated with primary antibodies against eIF5A2 (Abcam, Cambridge, MA, USA), Twist (Cell Signaling Technology, Danvers, MA, USA), E-cadherin (Cell Signaling Technology, Danvers, MA, USA), N-cadherin (Cell Signaling Technology, Danvers, MA, USA), β-catenin (Cell Signaling Technology Danvers, MA, USA), vimentin (Cell Signaling Technology, Danvers, MA, USA), and GAPDH (Cell Signaling Technology, Danvers, MA, USA) (dilution 1:2,000) at 4°C overnight, washed with TBS/T three times, and incubated with appropriate secondary antibodies (Cell Signaling Technology, Danvers, MA, USA) (dilution 1:2,000) for 2 h at room temperature. The protein bands were detected using ECL (GE Healthcare, Piscataway, NJ, USA) and visualized using film condensation exposure.

siRNA transfection and plasmid transfection assay

GC cells were transfected with eIF5A2 siRNA (Santa Cruz, Texas, USA) using Lipofectamine 2000 (Invitrogen, California, USA) according to the manufacturer's instructions. The sequences of siRNAs were as follows: si-eIF5A2 duplex sense, 5'-GCAGACGAAAUUGAUUUCATT-3' and anti-sense, 5'-UGAAAUCAAUUUCGUCUGCTT-3'; si-Twist duplex sense, 5'-GGUGUCUAAAUGCA-

UUCAUTT-3' and anti-sense, 5'-AUGAAUGCA-UUUAGACACCTT-3'. The transfection medium (Opti-MEM) (Gibco Company, Massachusetts, USA) was replaced with complete medium 6 h after transfection, and then the cells were incubated for 24 h. GC cells were transfected with 2 µg plasmid using Lipofectamine 2000 (Invitrogen, California, USA) according to the manufacturer's instructions. The transfected cells were harvested for further analysis after incubation for 6-8 hours at 37°C.

Immunofluorescence analysis

Cells were incubated with primary antibodies against E-cadherin (Abcam, Cambridge, MA, USA, 1:200) overnight at 4°C and then incubated with FITC-conjugated goat antibodies against rabbit or mouse IgG. The coverslips were counterstained with DAPI (Sigma, D9542, USA) and then imaged with a confocal laser scanning microscope (Olympus, Tokyo, Japan).

Statistical analysis

The experimental values were expressed as the mean ± SD. Data were analyzed using the SPSS statistical software (standard version SPSS 23.0). Two groups were analyzed with the Student's t test, and multiple group comparisons were conducted using one-way analysis of variance (ANOVA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

eIF5A2 expression levels in GC cell lines are negatively correlated with sensitivity to cisplatin

We conducted Western blot analysis to investigate the expression status of eIF5A2 in various GC cell lines and to investigate the effect of cisplatin on expression of eIF5A2. The results showed that the expression levels of eIF5A2 protein differed in GC cells with different degrees of differentiation, with the well-differentiated MGC-803 cells expressing the least amount of eIF5A2 protein (**Figure 1A**). The expression of eIF5A2 was upregulated to varying degrees in various cell lines after cisplatin treatment (**Figure 1D**). The sensitivity of each GC cell line to cisplatin was detected by CCK-8 assay (**Figure 1B**). The results showed that GC cells with different degrees of differentiation
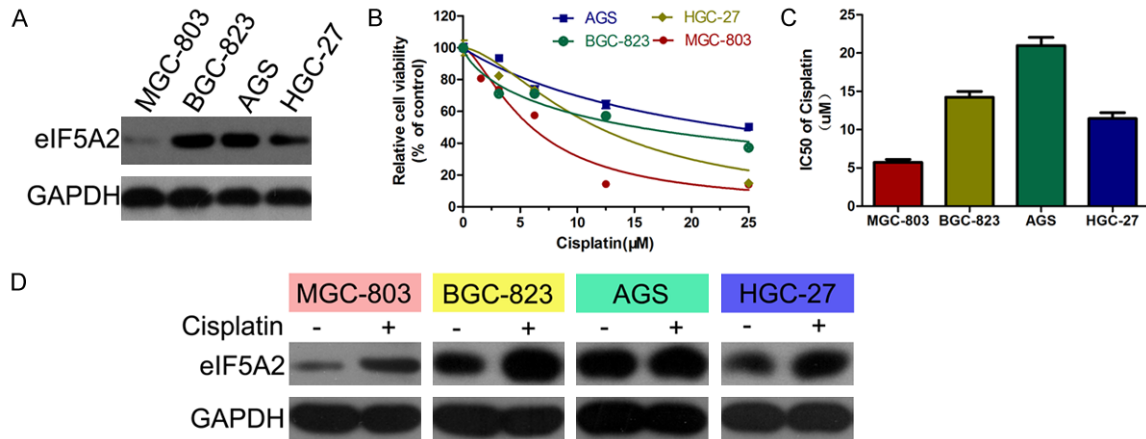
Figure 1. Expression of eIF5A2 in GC cell lines and sensitivity of GC cells to cisplatin. A. Western blot was used to detect the expression of eIF5A2 in GC cell lines. GAPDH was used as the internal control. B, C. GC cell lines were treated with different concentrations of cisplatin (0, 3.125, 6.25, 12.5, 25 μmol/L). Cell viability was examined using the CCK-8 assay. D. Western blot was used to detect the effect of cisplatin to the expression of eIF5A2 in GC cell lines. GAPDH was used as the internal control.

have different susceptibilities to cisplatin. Among them, well-differentiated MGC-803 cells were most sensitive to cisplatin (**Figure 1B**, **1C**).

eIF5A2 regulates the sensitivity of GC cells to cisplatin

To investigate the relationship between eIF5A2 and the sensitivity of GC cells to cisplatin, we conducted CCK-8 assay after transfecting with si-eIF5A2 or eIF5A2-overexpressing plasmid in the presence of cisplatin. The results showed that the sensitivity of cells to cisplatin was increased after siRNA-mediated silencing of eIF5A2 expression (**Figure 2A-D**), and was reduced with eIF5A2 overexpression (**Figure 2E**, **2F**). The transfection efficiencies of si-eIF5A2 and eIF5A2-overexpression plasmids were detected using Western blot (**Figure 2I**, **2J**).

EMT regulates the sensitivity of GC cells to cisplatin

To assess whether EMT plays a role in the sensitivity of GC cells to cisplatin treatment, we evaluated the expression of EMT-related markers in GC cells by Western blot. After treatment with cisplatin, the expression of epithelial markers E-cadherin and β-catenin was decreased, while the expression of Twist, vimentin, and N-cadherin were upregulated. However, Twist knockdown using si-Twist blocked these changes (**Figure 3A**). Meanwhile, CCK-8 experiments showed that si-Twist can enhance the sensitivi-

ty of GC cells to cisplatin (**Figure 3B**). The transfection efficiency of siRNA-Twist was detected using Western blot (**Figure 3C**). EDU analysis showed that si-Twist could enhance the inhibitory effect of cisplatin on the proliferation of GC cells (**Figure 3D**, **3E**).

eIF5A2 regulates EMT

To examine the effect of eIF5A2 on EMT in GC cells, the expression of EMT markers were analyzed by Western blot. As described previously, Western blot experiments showed that the expression of E-cadherin and β-catenin decreased after cisplatin treatment of GC cells, while the expression of eIF5A2, vimentin, and N-cadherin increased, suggesting that EMT occurred in GC cells. However, treatment with si-eIF5A2 blocked this effect (**Figure 4A**, **4B**). This indicates the upregulation of eIF5A2 leads to EMT. As described above, EMT regulates the sensitivity of GC cells to cisplatin. Immunofluorescence results showed that after si-eIF5A2 treatment, it could significantly increase the inhibitory effect of cisplatin on various GC cells (**Figure 4C**). Together, these data suggest that eIF5A2 regulates the sensitivity of GC cells to cisplatin by regulating EMT progression.

Inhibition of EMT progression abolishes the effect of eIF5A2 on cisplatin resistance

We used CCK-8 assay to examine the effect of eIF5A2 on the sensitivity of GC cells to cisplatin
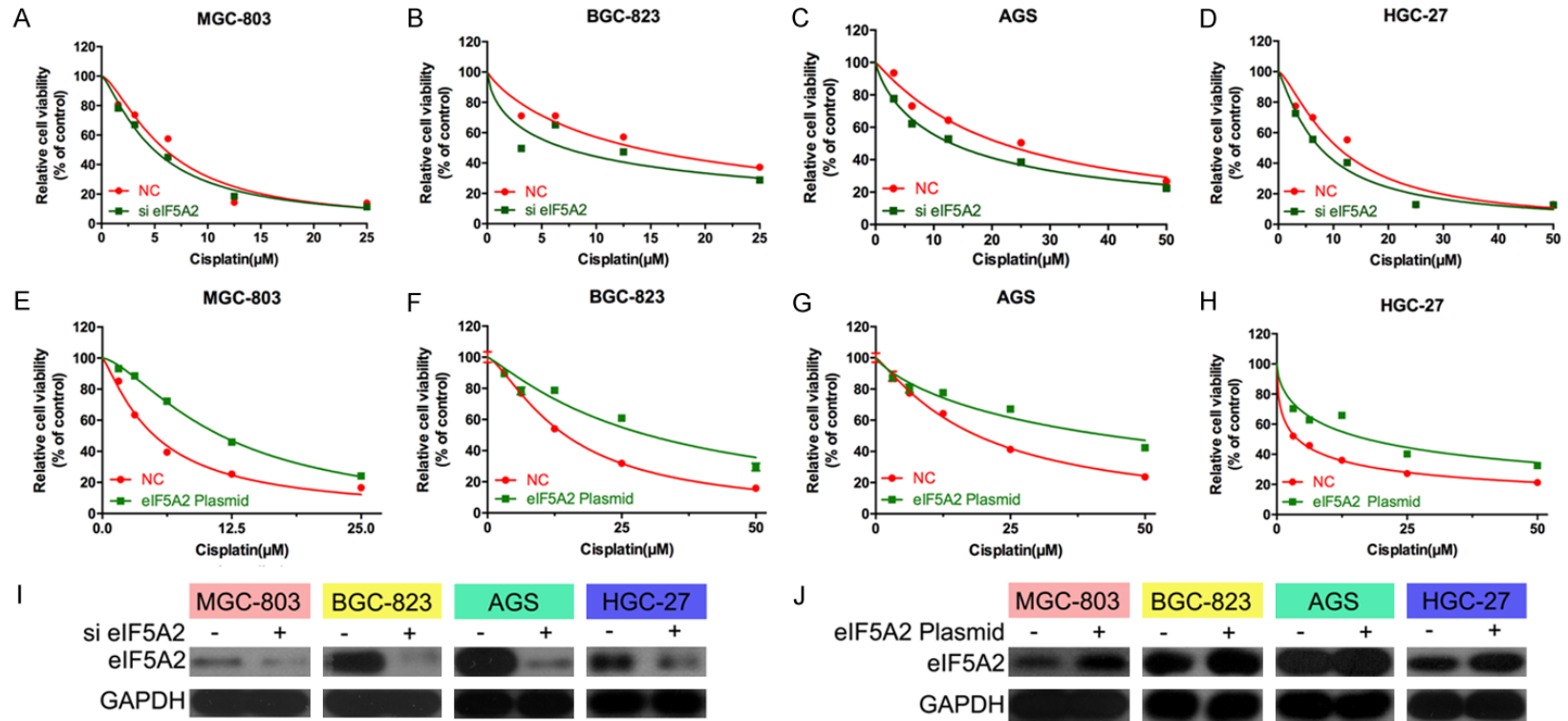
Figure 2. eIF5A2 expression levels and correlation with sensitivity to cisplatin. A-D. GC cell lines were treated with different concentrations of cisplatin (0, 3.125, 6.25, 12.5, 25, 50 µmol/L) and/or si-eIF5A2 (100 nmol/l). Cell viability was examined using the CCK-8 assay. E-H. GC cell lines were treated with different concentrations of cisplatin (0, 3.125, 6.25, 12.5, 25, 50 µmol/L) and/or eIF5A2 plasmid (2 µg/ml). Cell viability was examined using the CCK-8 assay. I, J. Western blot was used to detect the interference effciency of si-eIF5A2 and eIF5A2 overexpression plasmids in GC cell lines. GAPDH was used as the internal control.
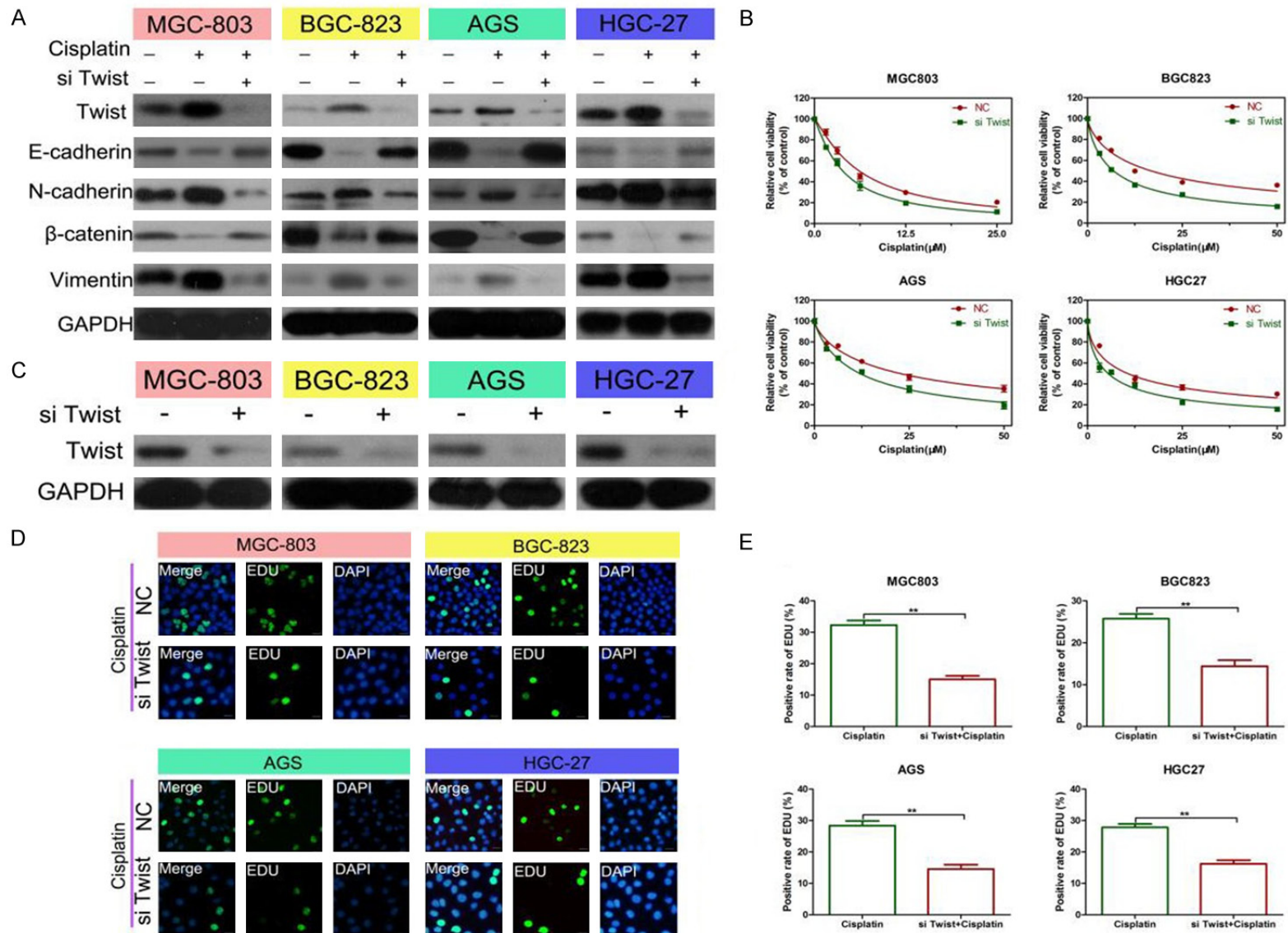
Figure 3. EMT and correlation with sensitivity to cisplatin. A. Western blotting of the effect of cisplatin and/or Twist siRNA on Twist, E-cadherin, N-cadherin, vimentin and β-catenin protein expression in GC cell lines. GAPDH was used as the internal control. B. GC cell lines were treated with different concentrations of cisplatin (0, 3.125, 6.25, 12.5, 25, 50 μmol/L) and/or Twist siRNA (2 μg/ml). Cell viability was examined using the CCK-8 assay. C. Western blot was used to detect the interference effciency of Twist siRNA in GC cell lines. GAPDH was used as the internal control. D, E. GC cell lines were treated with cisplatin (0, 3.125, 6.25, 12.5, 25, 50 μmol/L) and/or Twist siRNA (2 μg/ml). Cell proliferation was examined using the EDU assay; the numbers of EDU positive blotting confirmed Twist siRNA effectively downregulated protein expression.
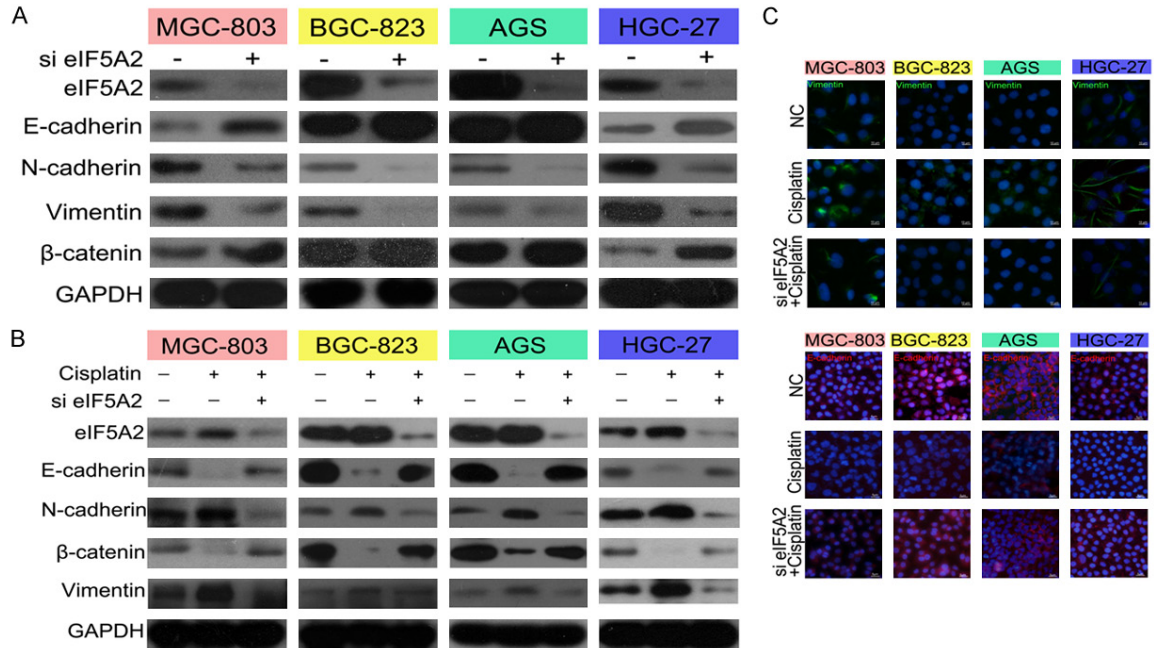
Figure 4. eIF5A2 expression and correlation with EMT-related markers. A. Western blotting of the effect of si-eIF5A2 on eIF5A2, E-cadherin, N-cadherin,vimentin and β-catenin protein expression in GC cell lines. GAPDH was used as the internal control. B. Western blotting of the effect of cisplatin and/or si-eIF5A2 on eIF5A2, E-cadherin, N-cadherin,vimentin and β-catenin protein expression in GC cell lines. GAPDH was used as the internal control. C. Immunofluorescence assay of the effect of cisplatin and/or si-eIF5A2 on vimentin (Green) and E-cadherin (Red) protein expression in GC cell lines; nuclei were stained using DAPI (blue).

after treatment with si-Twist. The results showed that after Twist inhibition, si-eIF5A2 treatment did not affect the sensitivity of GC cells to cisplatin, and there was no statistically significant difference compared with the control group (*P < 0.05) (**Figure 5A**). The transfection efficiencies of si-Twist, si-eIF5A2, and eIF5A2 overexpression plasmid were examined by Western blot (**Figure 5B-D**). The sensitivity of GC cells to cisplatin after treatment with si-Twist and eIF5A2 overexpression plasmid was detected by CCK-8 assay. The results showed that after Twist inhibition, eIF5A2 overexpression did not affect the sensitivity of GC cells to cisplatin, and there was no statistically significant difference compared with the control group (*P < 0.05) (**Figure 5E**).

Discussion

Gastric cancer is a major focus of cancer prevention and treatment in China. Cisplatin is the first-line agent for the treatment of GC; however, the propensity for gastric cancer to develop chemotherapy resistance remains a major limitation. EMT is thought to be the cause of increased invasion and metastasis of epithelial cancer cells, and it plays an important role in the development of chemotherapeutic resistance to a number of drugs including cisplatin [18, 19], paclitaxel [23], and 5-fluorouracil [24], yet its mechanism has not yet been fully elucidated.

Cytogenetic studies have found that chromosome 3q26 often exhibits abnormal amplification or instability in several human tumors, suggesting that there may be oncogenes in this region. In 2001, Guan et al. cloned a new gene, eIF5A2, from the 3q26 high-amplification region of human ovarian cancer, suggesting that eIF5A2 may be a tumor formation and metastasis gene. In-depth studies found that eIF5A2 is a homolog of eIF5A1 [25, 26], but eIF5A2 is specifically expressed only in some tumor cell types (such as bladder cancer [27] and colon cancer [17]), suggesting that eIF5A2 has tumor-specific expression characteristics and functions.

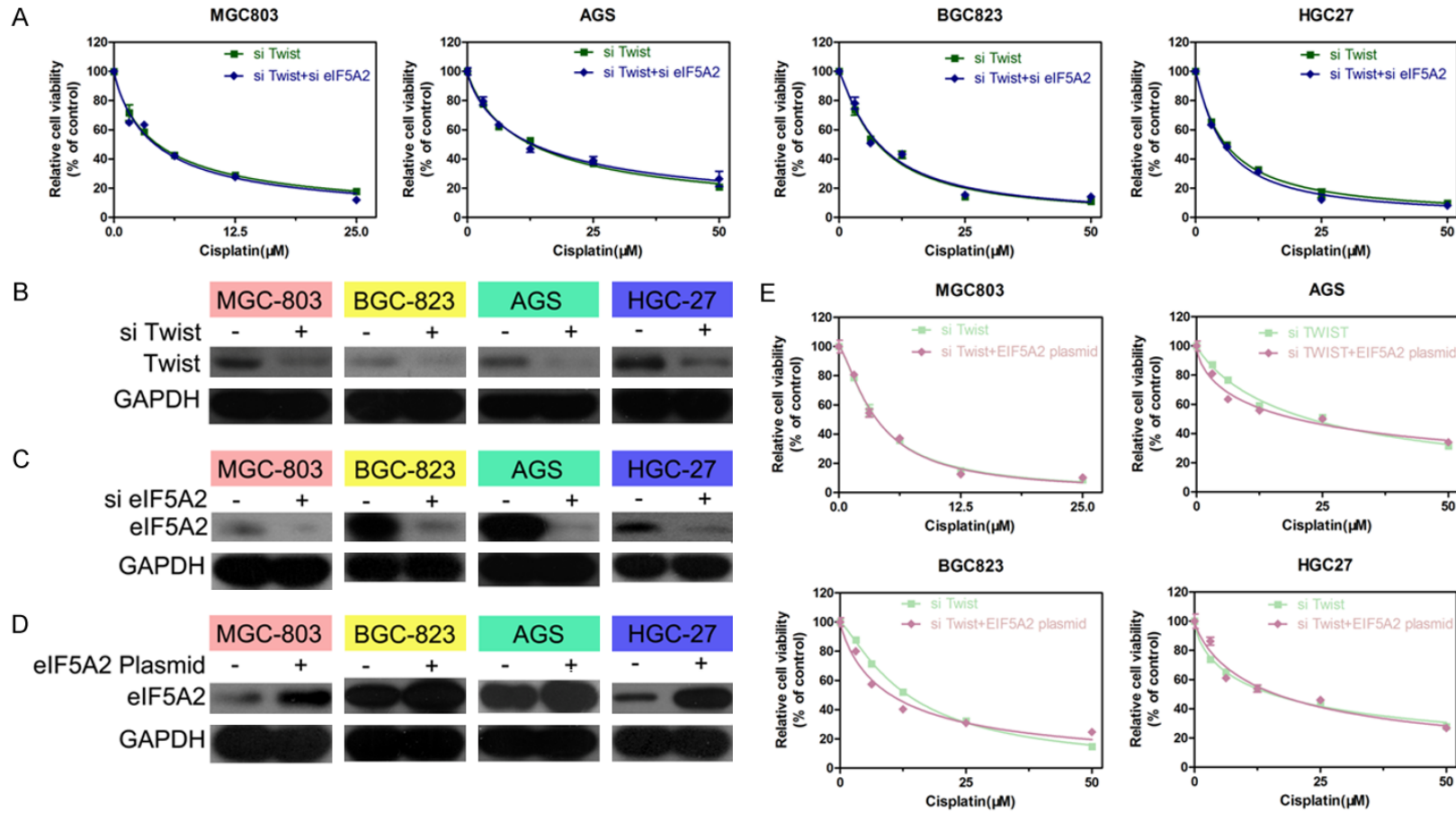Animal experiments have shown that overexpression of eIF5A2 causes proliferation of can-

Figure 5. EMT and correlation with the effect of eIF5A2 on cisplatin resistance. A. GC cell lines were treated with different concentrations of Twist siRNA and/or si-eIF5A2. Cell viability was examined using the CCK-8 assay. B-D. estern blot was used to detect the interference effciency of Twist siRNA, si-eIF5A2 and eIF5A2 overexpression plasmid in GC cell lines. GAPDH was used as the internal control. E. GC cell lines were treated with different concentrations of Twist siRNA and/or eIF5A2 plasmid. Cell viability was examined using the CCK-8 assay.

cer cells and tumorigenesis in nude mice [28]. In our study, eIF5A2 expression was found in all gastric cancer cell lines. Among them, undifferentiated HGC-27 cells and poorly differentiated BGC-823 and AGS cells expressed high levels of eIF5A2 protein and showed low sensitivity to cisplatin. Interestingly, the well-differentiated MGC-803 cell showed the opposite. These data suggest that higher expression of eIF5A2 protein is present in gastric cancer cells with higher malignancy, and that this may affect the sensitivity of gastric cancer cells to cisplatin. At the same time, we found that eIF5A2 is upregulated in gastric cancer cells after treatment with cisplatin, and that eIF5A2 knockdown via transfection of si-eIF5A2 can increase the sensitivity of gastric cancer cells to cisplatin. Altogether, these data demonstrate that eIF5A2 plays an important role in the inhibition of proliferation of gastric cancer cells by cisplatin.

Studies have shown that gastric cancer cells are prone to EMT during chemotherapy, and that this transition affects the therapeutic effect of anti-cancer agents. We hypothesized that the occurrence of EMT may also affect the sensitivity of gastric cancer cells to cisplatin. Therefore, we examined changes in expression of EMT-related markers following treatment of gastric cancer cells with cisplatin. The results showed that E-cadherin and β-catenin protein decreased after cisplatin treatment, while Twist, vimentin and N-cadherin proteins increased, suggesting that EMT had occurred. Twist regulates multiple gene promoters and as an EMT-inducible transcription factor it is critical for controlling EMT progression [29-31]. Our data shows that the EMT process can be blocked and the sensitivity of gastric cancer cells to cisplatin can be increased by inhibiting the expression of Twist, indicating that EMT regulates the sensitivity of gastric cancer cells to cisplatin. We hypothesized that the resistance of eIF5A2 to cisplatin in gastric cancer cells may also be related to the EMT process of gastric cancer cells. The results also showed that after si-eIF5A2-mediated knockdown of eIF5A2, epithelial markers such as E-cadherin and β-catenin were upregulated, while the expression of mesenchymal markers vimentin and N-cadherin decreased, indicating that si-eIF5A2 can reverse the EMT process of cells and block the effect of cisplatin on EMT-related markers. We found that si-eIF5A2-mediated knockdown of eIF5A2 and eIF5A2 plasmid overexpression did not affect the sensitivity of gastric cancer cells to cisplatin after si-Twist was used to block EMT progression. This suggests that eIF5A2 regulates the resistance of gastric cancer cells to cisplatin by mediating EMT.

Altogether, our data come to the conclusion that eIF5A2 may be a novel molecular target for overcoming chemoresistance. It can provide a more comprehensive treatment strategy for gastric cancer, which has important clinical significance for judging the clinical prognosis of gastric cancer and improving the quality of life.

Acknowledgements

Disclosure of conflict of interest

None.

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