

Original Article

MicroRNA-613 induces the sensitivity of gastric cancer cells to cisplatin through targeting SOX9 expression

Minghui Xue¹, Guangyan Li², Peisheng Sun¹, Dezhong Zhang¹, Xiangjie Fang¹, Wei Li³

Departments of ¹Gastrointestinal Surgery, ²Gastroenterology, ³Scientific Research and Postgraduate Education, The First Affiliated Hospital of Xinxiang Medical University, Weihui 453100, Henan, China

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Abstract: Increasing evidences have suggested that deregulated miRNAs may involve in drug chemoresistance in a lot of human cancers. However, the role of miR-613 in drug chemoresistance of GC cell is still unknown. The expression of miR-613 and Sex-determining region Y (SRY)-box 9 (SOX9) in GC tissues and cell lines was detected by using qRT-PCR. Cell migration and viability were measured by the wound healing assay and CCK-8 assays. Western blot and dual-luciferase reporter were done to identify the target gene of miR-613. We showed that miR-613 expression was downregulated in GC tissues and cell lines. Ectopic expression of miR-613 increased the sensitivity of GC cells to cisplatin. Overexpression of miR-613 suppressed GC cell proliferation, cycle and migration. In addition, we identified SOX9 was a direct target gene of miR-613 in GC cell. We showed that SOX9 expression was upregulated in gastric cancer samples. Moreover, the expression of SOX9 was negatively correlated with miR-613 expression in GC tissues. Furthermore, elevated expression of miR-613 increased the sensitivity of GC cells to cisplatin and suppressed GC cell proliferation and migration by targeting SOX9. These data suggested that miR-613 might function as a chemoresistant suppressor in GC.

Keywords: Gastric cancer, miRNAs, miR-613, SOX9, cisplatin

Introduction

Gastric cancer (GC) is the fifth most common tumor and is the third leading cause of death from tumor in the world [1-4]. For most solid malignancies, recurrence and metastasis are the predominant obstacles to the cure of GC [5, 6]. The incidence of GC and resulting mortality are decreased due to improvements in treatment and diagnosis and better living conditions [7-9]. GC is usually diagnosed at the advanced stage because of lacking early diagnostic markers [10-13]. Therefore, it is important to study the molecular mechanism underlying GC initiation and development, as well as to find novel diagnosis makers and therapeutic targets for GC.

MicroRNAs (miRNAs) are a group of small non-protein-coding RNAs that inhibit protein translation by binding to 3'untranslated (3'UTR) regions of target mRNAs [14-16]. Emerging studies have shown that deregulated expression of miRNAs is found in a lot of tumors and was

associated with cancer initiation and progression [17-21]. MiRNA are associated with many cell processes such as cell development, proliferation, metabolism, differentiation, migration and invasion [22-24]. Recently, several evidences have indicated that deregulated miRNAs may involve in drug chemoresistance in a lot of human cancers [6, 9, 25]. Several studies reported that miR-613 acted important roles in a lot of tumors [26-29]. For instance, Zhang et al indicated that miR-613 expression was decreased in the retinoblastoma cell lines and tissues. Overexpression of miR-613 suppressed the retinoblastoma cell invasion, migration and proliferation and induced cell cycle arrest via suppressing E2F5 expression. Li et al [30]. reported that miR-613 expression level was downregulated in the colorectal cancer (CRC) tissues and cell lines. Ectopic expression of miR-613 suppressed CRC cell migration, invasion and proliferation and cell cycle through targeting the FMNL2 expression. Li et al [31]. showed that the expression level of miR-613 was decreased in osteosarcoma cell lines and

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tissues. Elevated expression of miR-613 suppressed the osteosarcoma cell proliferation and invasion by regulating the epithelial transition factor (c-MET) expression. However, the role of miR-613 in GC remains unknown.

A number of evidences have indicated that miR-613 played important roles in several tumors [26-29]. However, the role of miR-613 in GC is still unknown. In this study, we tried to study the expression of miR-613 in GC tissues and cell lines. We found that miR-613 expression was downregulated in GC tissues and cell lines. Ectopic expression of miR-613 increased the sensitivity of GC cells to cisplatin. Overexpression of miR-613 suppressed GC cell proliferation, cycle and migration. In addition, we identified Sex-determining region Y (SRY)-box 9 (SOX9) was a direct target gene of miR-613 in GC cell.

Materials and methods

Tissue samples

Fresh tissues from GC and the corresponding normal adjacent sample were collected from 40 cases at The First Affiliated Hospital of Xinxiang Medical University between 2014 and 2016. The tissues were snap-frozen in the liquid nitrogen and stored until RNA extraction. Approval to do this study was obtained from Institutional Review Board of The First Affiliated Hospital of Xinxiang Medical University and every patient has written informed consent.

Cell culture and transfection

Human GC cell lines (MGC-803, HGC-27, HGC-27 and SGC-7901) and normal gastric epithelial cell line (GES-1) were purchased from the cell bank of Chinese Academy of Medical Sciences (Beijing, China) and were cultured in the Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with fetal bovine serum (FBS) (Gibco, USA), penicillin and streptomycin. miR-613 mimics and the scramble mimics were synthesized from GenePharma (Shanghai, China). Cell transfection was performed with Lipofectamine 2000 (Life Technologies, Inc.) following to instruction.

Quantitative real-time PCR

Total RNA was isolated from tissues and cells by using the Trizol kit (Invitrogen). Quantitative

real-time PCR (qRT-PCR) was conducted with SYBR (TaKaRa) and measured by using the ABI 7500 Real-Time PCR system (Applied Biosystems, USA). The relative expression was measured by using the $2^{-\Delta\Delta Ct}$ method. RNA U6 was performed as the internal control for miRNA and GAPDH was used for mRNA expression. The Primers were used as follows: SOX9, 5'-CAGAACCACAGCAGTTAA-3' (forward), 5'-AA-CAACAGATGAC CATAACC-3'; GAPDH, 5'-CGGAGTCAACGGATTGGTCGTAT-3' (forward), 5'-AG-CCTTCTCCATGGTGGTGAAGAC-3' (reverse).

Cell proliferation and cell cycle and migration

Cell proliferation was assessed with the Cell Counting kit (CCK)-8 assay following to the manufacturer's information. Cells were cultured in the 96-well plates. Absorbance was determined at the 450 nm by using the microplate reader. Cell cycle was determined using propidium iodide (PI) solution containing RNase (Beyotime, China) for staining, and caclutated using FACScan cytofluorometry (Becton Dickinson, USA). For cell migration, wound healing assay was performed. Cells were plated in the 6-well plate and cultured to near confluence (>90%) or confluence. A sterile pipette tip was used to make a wound through the cell layer. The cell wound was photographed at the time 0 and 48 hours.

Western blotting

Total cell or tissue extract was prepared using Protein Extraction kit (KangChen Bio-Tech, China) according to manufacturer's information. Total protein was separated through denaturing 12% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After blocking with 5% bovine serum albumin, the membranes were incubated with SOX9 antibody (Cell Signaling Technology, USA). The membrane was visualized by an enhanced chemiluminescence kit (ECL) (Beyotime Institute of Biotechnology).

Dual luciferase assays

The 3'UTR of SOX9 cDNA containing target site of the miR-613 was synthesized and then inserted into the pGL3-control vector (Promega, WI) at the XbaI site. miR-613 overexpressing and scramble cells were cultured in the 24-well plate and transfected with the luciferase reporter vector and pRL-TK vector expressing

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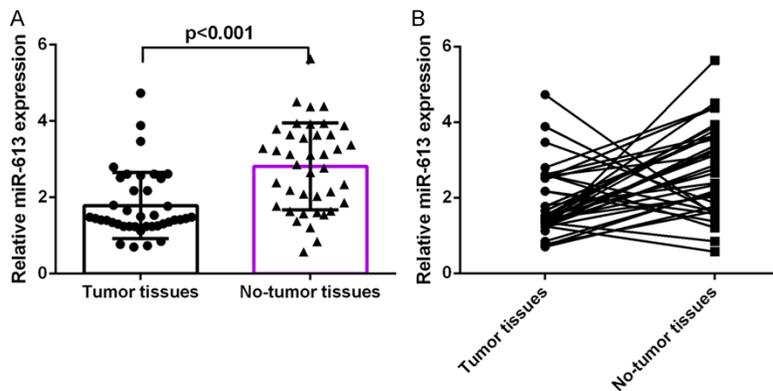


Figure 1. The expression of miR-613 was downregulated in the gastric cancer tissues. A. The expression of miR-613 in the GC tissues and corresponding normal adjacent samples was determined by qRT-PCR analysis. U6 was used as the internal control. B. miR-613 expression was downregulated in 31 (31/40, 77.5%) GC patients compare to the adjacent non-tumor tissues.

Renilla luciferase (Promega, Madison, USA) by using Lipofectamine 2000 reagent (Life Technologies, Inc.). After 48 hours, the luciferase activity was determined using the Dual-Luciferase Reporter Assay (Promega, Madison, WI) following to the manufacturer's information.

Statistical analysis

The results were shown as the mean \pm SD. SPSS 17.0 software (SPSS, Chicago, USA) was performed to determine the data. Statistical difference was measured using analysis of variance (ANOVA) and Student's t-test. $P < 0.05$ was considered to be statistically significant.

Result

The expression of miR-613 was downregulated in gastric cancer tissues

We firstly determined miR-613 expression in GC samples. Our data indicated that miR-613 expression was lower in GC tissues than in the corresponding normal adjacent samples (**Figure 1A**). In addition, miR-613 expression was downregulated in 31 (31/40, 77.5%) GC cases compare to the adjacent non-tumor tissues (**Figure 1B**).

MiR-613 promoted the sensitivity of gastric cancer cells to cisplatin

The expression of miR-613 was downregulated in GC cell lines (MKN-45, MGC-803, HGC-27, and SGC-7901) compared with normal gastric

epithelial cell line (GES-1) (**Figure 2A**). The expression of miR-613 was significantly upregulated in GC cell line HGC-27 after treated with miR-613 mimic (**Figure 2B**). The response of the HGC-27 cell to cisplatin was increased after treated with miR-613 mimic compared with the scramble-transfected cells (**Figure 2C**).

SOX9 expression was up-regulated in gastric cancer samples

We next measured the SOX9 expression in GC tissues.

We found that SOX9 expression was higher in GC tissues than in the corresponding normal adjacent samples (**Figure 3A**). In addition, SOX9 expression was upregulated in 30 (30/40, 75%) GC cases compared to the adjacent non-tumor tissues (**Figure 3B**). Moreover, we showed that the expression of SOX9 was negatively correlated with the miR-613 expression in GC tissues (**Figure 3C**).

SOX9 was a direct target gene of miR-613

We then utilized bioinformatic system (<http://www.targetscan.org>) to find the potential target of miR-613. MiR-613 and its potential putative binding sequence in the 3'-UTR of SOX9 was shown in **Figure 4A**. Overexpression of miR-613 decreased the activity of firefly luciferase which carried the wild-type (WT) but not mutant (Mut) 3'-UTR of SOX9 (**Figure 4B**). Ectopic expression of miR-613 suppressed SOX9 protein expression in HGC-27 cell (**Figure 4C**).

Ectopic expression of miR-613 suppressed gastric cancer cell proliferation, cycle and migration

Elevated expression of miR-613 decreased HGC-27 cell proliferation by using CCK-8 assay (**Figure 5A**). In addition, we indicated that overexpression of miR-613 suppressed HGC-27 cell cycle (**Figure 5B**). Moreover, we showed that ectopic expression of miR-613 suppressed the ki-67 expression in the HGC-27 cell (**Figure 5C**). Overexpression of miR-613 inhibited the cyclin D1 expression in the HGC-27 cell (**Figure 5D**). In

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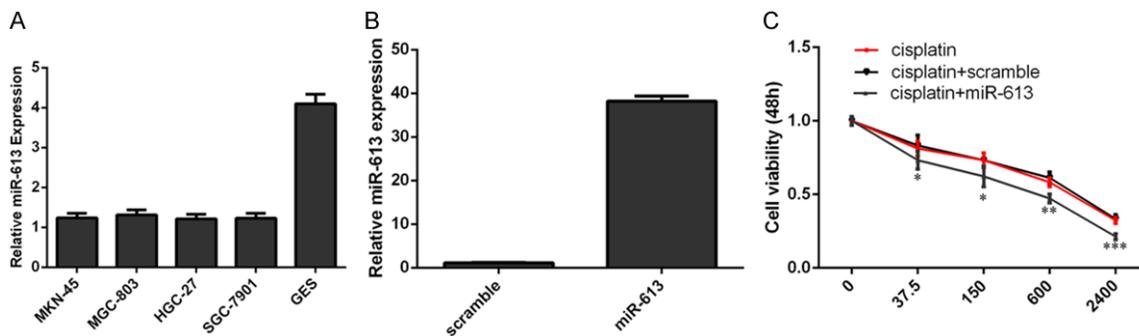


Figure 2. miR-613 promoted the sensitivity of gastric cancer cells to cisplatin. A. The expression level of miR-613 in the GC cell lines (MKN-45, MGC-803, HGC-27, and SGC-7901) and normal gastric epithelial cell line (GES-1) was detected by qRT-PCR. U6 was used as the internal control. B. The expression of miR-613 in the GC cell line HGC-27 after treated with miR-613 mimic was measured by qRT-PCR. Overexpression of miR-613 significantly increased miR-613 expression in the HGC-27 cell. C. The response of the HGC-27 cell to cisplatin was increased after treated with miR-613 mimic compared with the scramble-transfected cells. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

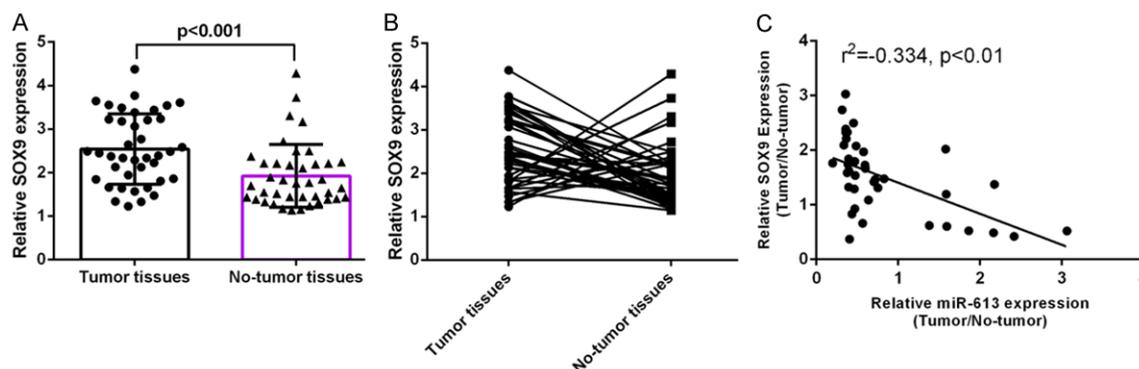


Figure 3. SOX9 expression was upregulated in the gastric cancer samples. A. The expression level of SOX9 in the GC tissues and corresponding normal adjacent samples was determined by qRT-PCR. GAPDH was used as the internal control. B. SOX9 expression was upregulated in 30 (30/40, 75%) GC cases compare to the adjacent non-tumor tissues. C. The expression of SOX9 was negatively correlated with the miR-613 expression in the GC tissues. The relationship between expression of miR-613 and SOX9 was determined by Spearman correlation analysis.

addition, we showed that ectopic expression of miR-613 inhibited HGC-27 cell migration and the relative open wound was shown in **Figure 5E** and **5F**.

miR-613 promoted the sensitivity of gastric cancer cells to cisplatin and suppressed gastric cancer cell proliferation and migration by targeting SOX9

Next, we overexpressed SOX9 expression by pcDNA-SOX9 vector to determine whether SOX9 was involved in the antitumor roles of miR-613. The expression of SOX9 were significantly upregulated in HGC-27 cell after treated with pcDNA-SOX9 vector (**Figure 6A** and **6B**). The response of the HGC-27 cell to cisplatin was decreased after treated with pcDNA-SOX9

vector compared with the control vector-transfected cells (**Figure 6C**). Furthermore, after cotransfected with the pcDNA-SOX9 and miR-613 mimic, overexpression of SOX9 rescued the tumor suppressor effect of miR-613 in HGC-27 cell at the response to cisplatin (**Figure 6D**). In addition, ectopic expression of SOX9 rescued the tumor suppressor effect of miR-613 at cell proliferation (**Figure 6E**) and cell migration (**Figure 6F** and **6G**).

Discussion

GC is the fifth most common tumor and is the third leading cause of death from tumor in the world [16, 32-34]. Chemotherapeutic agents are widely used in GC treatment [9]. The mechanisms of chemo-drug action are to form inter-

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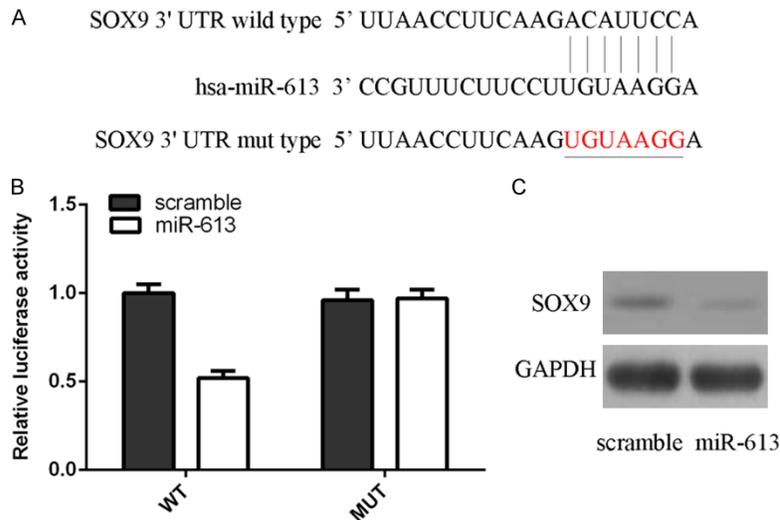


Figure 4. SOX9 was a direct target gene of miR-613. A. miR-613 and its potential putative binding sequence in the 3'-UTR of SOX9 was shown. B. Ectopic expression of miR-613 decreased the activity of firefly luciferase which carried the wild-type (WT) but not mutant (Mut) 3'-UTR of SOX9. C. Overexpression of miR-613 decreased the protein expression of SOX9 in the HGC-27 cell.

and intra-strand with cancer cell DNA and induce cancer apoptosis cisplatin to stand for a significant barrier to promote the long term overcome of GC patients [35, 36]. Factors that improve the sensitivity of GC cells to drugs may highlight predictive targets or biomarkers for treatment [6, 37]. Increasing evidences suggested that miRNAs played important roles in drug chemoresistance in GC cells [9, 38]. In the present study, we indicated that miR-613 expression was downregulated in GC tissues and cell lines. Ectopic expression of miR-613 increased the sensitivity of GC cells to cisplatin. Overexpression of miR-613 suppressed GC cell proliferation, cycle and migration. In addition, we identified SOX9 was a direct target gene of miR-613 in GC cell. We showed that SOX9 expression was upregulated in gastric cancer samples. Moreover, the expression of SOX9 was negatively correlated with miR-613 expression in GC tissues. Furthermore, elevated expression of miR-613 increased the sensitivity of GC cells to cisplatin and suppressed GC cell proliferation and migration by targeting SOX9. These data suggested that downregulation of miR-613 contributed to the modulation of cisplatin chemosensitivity and served as a tumor suppressor miRNA in GC.

A number of evidences have indicated that miR-613 played important roles in some tumors [26-29]. For example, Zhang et al showed that

miR-613 expression level was downregulated in retinoblastoma cell lines and tissues. Ectopic expression of miR-613 inhibited retinoblastoma cell invasion, migration and proliferation and promoted cell cycle arrest through inhibiting E2F5 expression. Li et al [30]. indicated that miR-613 expression was downregulated in colorectal cancer (CRC) tissues and cell lines. Overexpression of miR-613 inhibited CRC cell migration, invasion and proliferation and cell cycle by targeting the FMNL2. Li et al [31]. found that miR-613 expression was downregulated in osteosarcoma cell lines and tissues. Ectopic expression

of miR-613 inhibited osteosarcoma cell proliferation and invasion through targeting the epithelial transition factor (c-MET). Qiu et al [39]. showed that miR-613 expression level was downregulated in the papillary thyroid carcinoma tissues and cell lines and elevated expression of miR-613 inhibited the papillary thyroid carcinoma cell proliferation, invasion and migration by regulating sphingosine kinase 2 (SphK2) expression. However, the role of miR-613 in GC was still uncovered. In the present study, we firstly determined the expression of miR-613 in GC tissues. We found that the expression of miR-613 was downregulated in GC tissues and cell lines. Overexpression expression of miR-613 increased the sensitivity of GC cells to cisplatin and suppressed GC cell proliferation, cycle and migration. This data suggested that miR-613 played as a tumor suppressor miRNA in GC.

SOX9 is one member of the SOX (sry-related high-mobility group (HMG) box) transcription factor family [40, 41]. SOX9 is found in almost tissues during embryogenesis such as neural crest, cartilage, kidney, notochord and pancreas [42, 43]. Increasing evidences have suggested that SOX9 expression is upregulated in several tumors such as osteosarcoma, breast cancer, lung cancer, prostate cancer, colorectal cancer and also GC [41, 44-47]. Overexpression of SOX9 promoted cancer cell proliferation,

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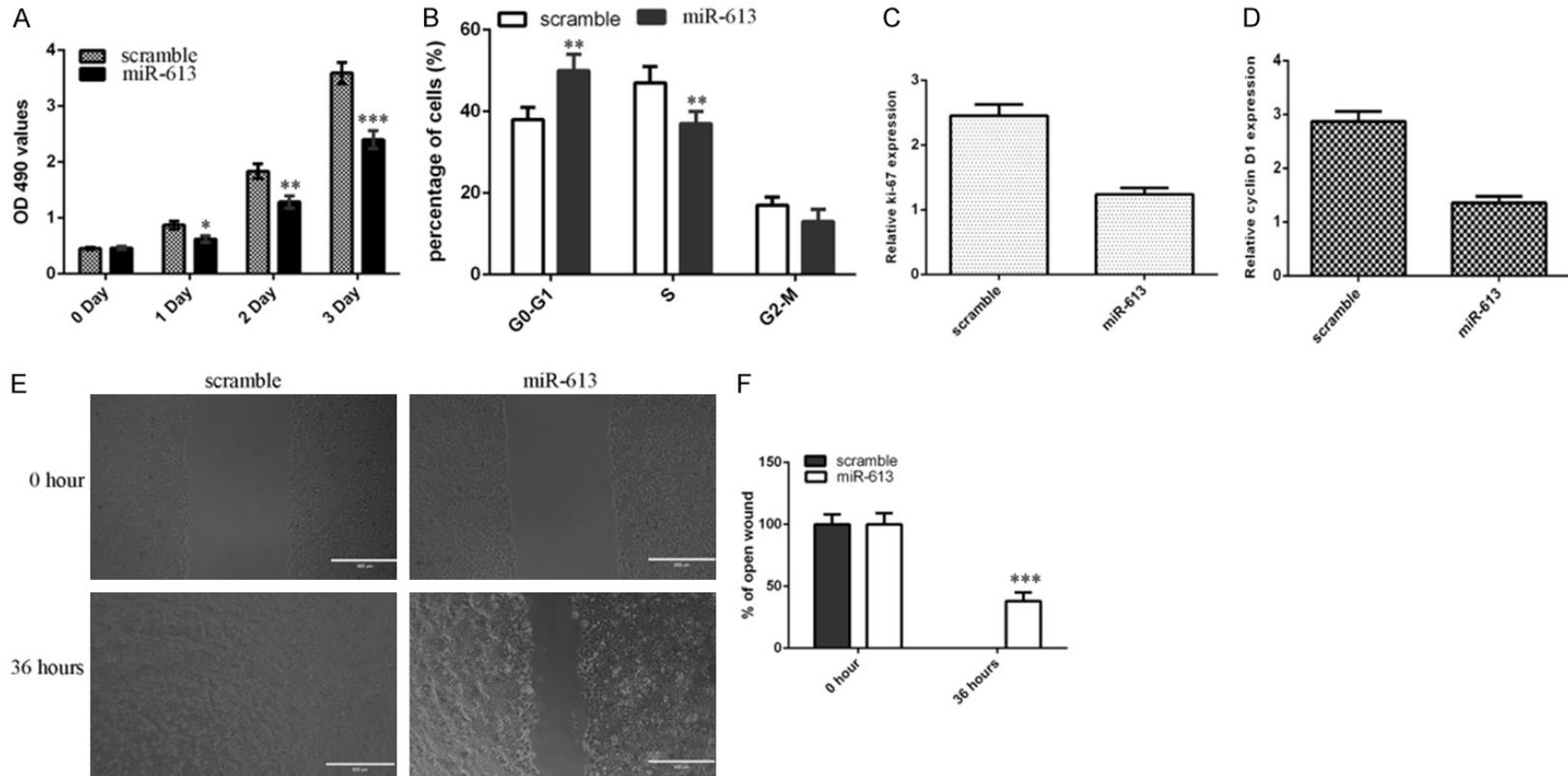


Figure 5. Ectopic expression of miR-613 suppressed the gastric cancer cell proliferation, cycle and migration. A. Elevated expression of miR-613 suppressed the HGC-27 cell growth. CCK-8 assay was performed to detect the cell proliferation. B. Ectopic expression of miR-613 inhibited the HGC-27 cell cycle. C. The expression of ki-67 was measured by qRT-PCR analysis. GAPDH was used as the internal control. D. Overexpression of miR-613 suppressed the cyclin D1 expression in the HGC-27 cell. E. Overexpression of miR-613 suppressed the HGC-27 cell migration. F. The relative open wound in different was shown. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

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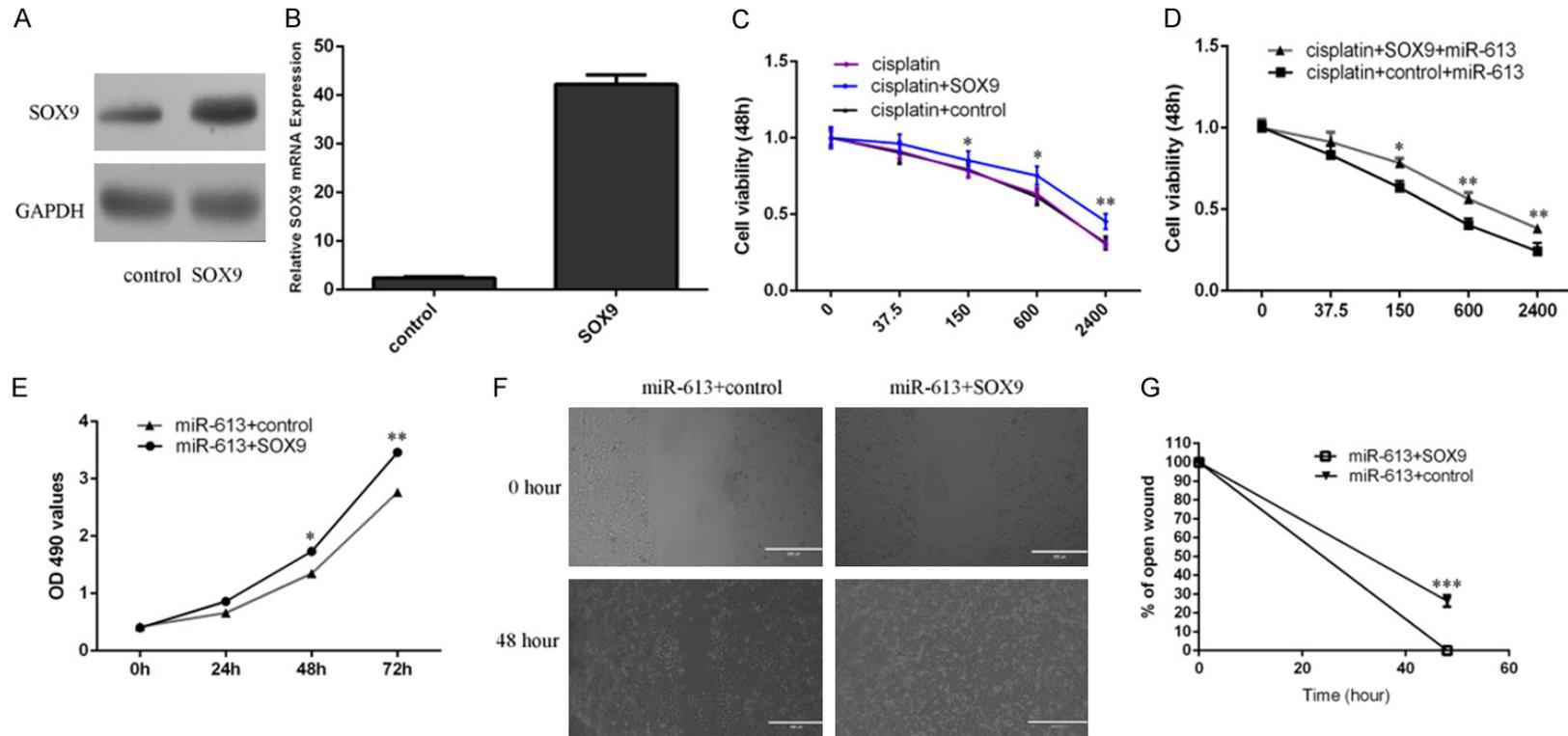


Figure 6. MiR-613 promoted the sensitivity of gastric cancer cells to cisplatin and suppressed the gastric cancer cell proliferation and migration by targeting SOX9 expression. A. The protein expression of SOX9 was measured by Western blot. B. The mRNA expression of SOX9 was significantly upregulated in the HGC-27 cell after treated with pcDNA-SOX9 vector. C. The response of the HGC-27 cell to cisplatin was decreased after treated with pcDNA-SOX9 vector compared with the control vector-transfected cells. D. Overexpression of SOX9 reduced the tumor suppressor effect of miR-613 in the HGC-27 cell at the response to cisplatin. E. Ectopic expression of SOX9 reduced the tumor suppressor effect of miR-613 at cell proliferation. F. Overexpression of SOX9 reduced the tumor suppressor effect of miR-613 at cell migration. G. Relative ratio of wound closure per field was shown. *P<0.05, **P<0.01 and ***P<0.001.

invasion and migration. Recently, Wang et al [48], demonstrated that overexpression of miR-524-5p promoted cisplatin sensitivity of GC cell through regulating proliferation and metastasis by inhibiting SOX9 expression. In our study, we indicated that overexpression of miR-613 decreased the activity of firefly luciferase which carried the wild-type (WT) but not mutant (Mut) 3'-UTR of SOX9. Ectopic expression of miR-613 suppressed SOX9 protein expression in the GC cell. These results suggested that SOX9 was a direct gene of miR-613 in GC cell. In addition, we found that SOX9 expression was upregulated in GC samples. Moreover, the expression of SOX9 was negatively correlated with miR-613 expression in GC tissues. Furthermore, ectopic expression of miR-613 enhanced the sensitivity of GC cells to cisplatin and suppressed GC cell proliferation and migration by targeting SOX9.

In summary, we indicated that miR-613 was downregulated in GC cell lines and tissues and overexpression of miR-613 enhanced the sensitivity of GC cells to cisplatin and inhibited GC cell proliferation and migration by targeting SOX9. These data suggested that miR-613 might function as a chemoresistant suppressor in GC.

Disclosure of conflict of interest

None.

Address correspondence to: Minghui Xue, Department of Gastrointestinal Surgery, The First Affiliated Hospital of Xinxiang Medical University, Weihui 453100, Henan, China. E-mail: liyanling17@yeah.net

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