

Original Article

Long non-coding RNA GAS5 acts as a molecular sponge to regulate miR-23a in gastric cancer

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Abstract: Background: Long non-coding RNA (lncRNAs) play critical roles in the development of cancers. lncRNA GAS5 was identified to be involved in tumorigenesis of several cancers. However, its role and function in gastric cancer (GC) remains unknown. Methods: The expression of GAS5, miR-23a and MT2A in 24 paired GC tissues was detected by qRT-PCR and subjected to correlation analysis. Bio-informatics analysis was performed by using DIANA Tools. Abnormal GAS5 expression was conducted in GC cells to analyze its regulation on miR-23a and MT2A via using qRT-PCR, western blot and luciferase reporter assay. Results: We showed that GAS5 expression was decreased in GC tissue and inversely correlated with up-regulated expression of miR-23a. GAS5 negatively regulated miR-23a expression in GC cells. The bio-informatics prediction showed putative miR-23a binding sites within GAS5 transcripts. Furthermore, our data indicated the positive regulation of GAS5 on the miR-23a target, MT2A, wherein GAS5 suppressed the negative regulation of miR-23a on MT2A by binding its 3'UTR. Additionally, the expression of MT2A was also decreased in GC tissues, showing a positive or negative correlation with GAS5 or miR-23a, respectively. Conclusion: These observations suggested that GAS5 could act as a ceRNA play critical roles in GC pathogenesis and might serve as a potential therapeutic target for the treatment of gastric cancer.

Keywords: Gastric cancer, GAS5, miR-23a, MT2A

Introduction

Gastric cancer (GC) is one of the most common malignancies and the second leading cause of cancer deaths worldwide [1]. In spite of the improvement in the diagnosis and treatment of GC, the five-years overall survival rate remains lower than 30% due to delayed diagnosis, metastasis and recurrence [2, 3]. Although there is a great advancement on the gastric carcinogenesis, the molecular mechanisms underlying gastric cancer development and progression are still unclear [4]. Therefore, a better understanding of molecular mechanisms underlying gastric carcinogenesis will improve diagnosis and treatment of GC.

With the development of whole-genome sequencing technology, it was determined that protein-coding sequences occupy less than 2% of the human genome [5]. Long non-coding RNAs (lncRNAs) are a class of non-coding RNAs (ncRNA) that greater than 200 nucleotides in

length, with no potential protein translation capacity [6]. Recent evidences showed that lncRNAs participate in a large number of cellular processes, such as epigenetic regulation, alternative splicing, RNA decay, cell differentiation, cell cycle control, cancer cells metastasis and drug resistance [7, 8]. Multiple lines of evidence revealed that lncRNAs are closely associated with gastric cancer. For example, Li et al showed that lncRNA H19 was overexpression in GC and enhanced carcinogenesis and metastasis of GC [9]. Sun et al found that down-regulated lncRNA MEG3 was associated with poor prognosis and promoted cell proliferation in GC [10]. Liu et al revealed that lncRNA HOTAIR functioned as a competing endogenous RNA to regulate HER2 expression by sponging miR-331-3p in GC [11].

Growth arrest-specific transcript 5 (GAS5), which was previously shown to be consistently down-regulated and identified as a tumor-suppressor lncRNA in tumor progression [12]. For

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example, Yin et al showed that overexpression of lncRNA GAS5 inhibited cell proliferation and predicted a poor prognosis in patients with colorectal cancer [13]. Liu et al suggested that downregulation of GAS5 promoted bladder cancer cell proliferation partly by regulating CDK6 [14]. Recently, Sun et al showed that GAS5 was downregulated in GC tissues and cells, wherein it might function as a tumor suppressor by decrease gastric cancer cell proliferation and induce apoptosis [15]. However, the underlying mechanism of the tumor suppressive role of GAS5 in GC was still unclear.

miRNAs are small, ncRNAs of 19 to 24 nucleotides in length, which could bind at the 3'UTR of potentially hundreds of target genes with imperfect complementarity, resulting in degradation of target mRNAs and inhibition of translation [16]. Recent studies showed that miR-23a play critical roles in GC progression. For example, Ma et al reported that up-regulation of miR-23a promoted tumor progression and conferred poor prognosis in GC patients [17]. An et al found that miR-23a in amplified 19p13.13 loci targeted metallothionein 2A and promoted growth in gastric cancer cells [18]. Liu et al reported that miR-23a targeted interferon regulatory factor 1 and modulated cellular proliferation and paclitaxel-induced apoptosis in GC cells [19]. An increasing number of studies reported another novel function of lncRNAs in tumorigenesis by sponging specific miRNAs due to their direct interaction [20].

In this study, bio-informatics analysis showed a potential interaction between GAS5 and miR-23a, suggesting that GAS5 might function as a miR-23a sponge in GC. Therefore, the expression of GAS5 and miR-23a were detected in GC tissues, and the relationship between GAS5 and miR-23a were further analyzed. More experiments were performed to validate the functional role of GAS5 in the regulation of metallothionein 2A (MT2A) expression by sponging miR-23a in GC cells.

Materials and methods

Tissue collection

Gastric cancer specimens and the corresponding adjacent non-tumor tissues were obtained from The First Affiliated Hospital of Henan Polytechnic University between 2010 and 2011

with informed consent. The patients were diagnosed with gastric cancer based on histopathological evaluation, and no local or systemic treatment was conducted before surgery. The protocols used in the study were approved by the Research Ethics Committee of Henan Polytechnic University.

RNA extraction and qRT-PCR detection

Total RNA from specimens and cells was isolated with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. 1 µg RNA was reverse transcribed in a final volume of 20 µl using random primers under standard conditions for the PrimeScript RT reagent Kit (TaKaRa). SYBR Premix Ex Taq (TaKaRa) was used for Quantitative real-time PCR (qRT-PCR) assays, which was carried out on Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems). The PCR primers for GAS5 5'-CTTCTGGGCTCAAGTGATCCT-3' and 5'-TTGTGCCATGAGACTCCATCAG-3'; MT2A, 5'-TCGCCATGGATCCCAACTG-3' and 5'-AGGTTTGTGGAA-GTCGCGT-3'; GAPDH, 5'-ACCCACTCCTCCACCTTTGAC-3' and 5'-TGTTGCTGTAGCCAAATTCGTT-3'; Their relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method with GAPDH as the control. All results were obtained from at least three experiments with triplicate reactions.

Cell culture

Gastric cancer cell lines (BGC-823 and MGC-803) were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin in humidified air at 37°C with 5% CO₂.

Cell transfection

Plasmids for GAS5 overexpression, containing pcDNA-GAS5 and empty pcDNA vector, and small-interference RNAs (siRNAs) for reducing GAS5 expression were obtained as described in a previous study [15]. GAS5 mutant plasmid was obtained by site-directed mutagenesis, according to the bio-informatics analysis described below. miR-23a mimics and inhibitors were purchased from GenePharma

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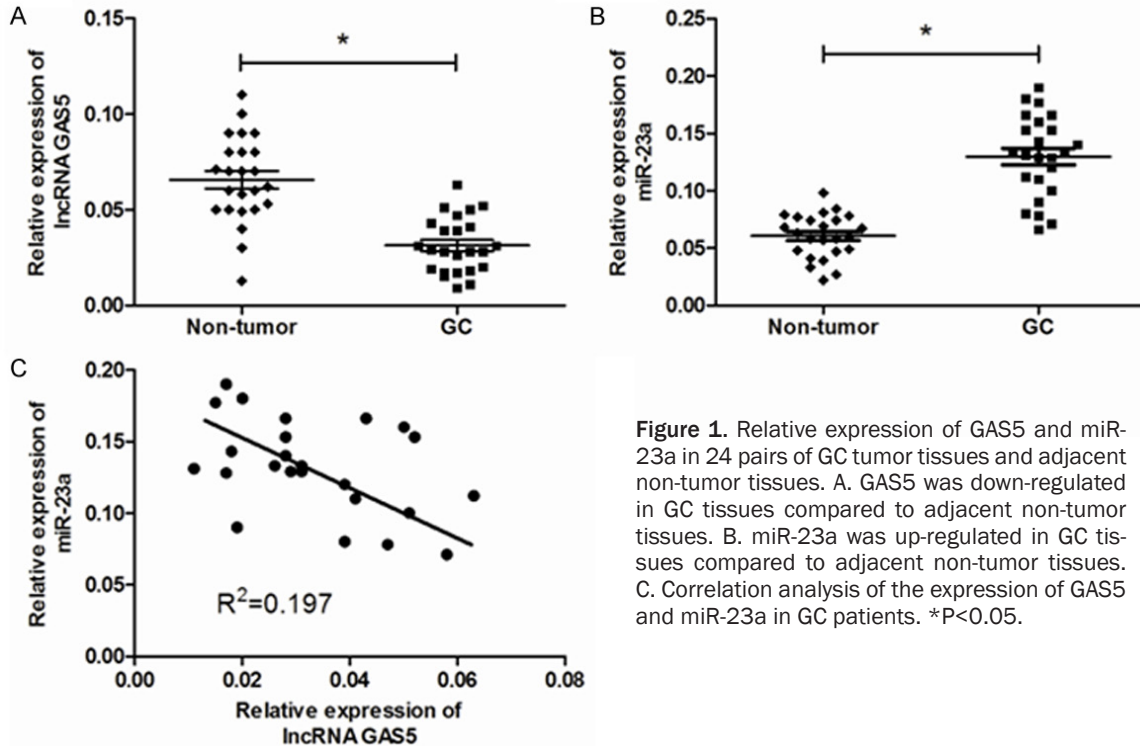


Figure 1. Relative expression of GAS5 and miR-23a in 24 pairs of GC tumor tissues and adjacent non-tumor tissues. A. GAS5 was down-regulated in GC tissues compared to adjacent non-tumor tissues. B. miR-23a was up-regulated in GC tissues compared to adjacent non-tumor tissues. C. Correlation analysis of the expression of GAS5 and miR-23a in GC patients. * $P<0.05$.

(Shanghai). GC cells were grown in 6-well plates and transfected by Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. At 48 h post-transfection, cells were harvested for further analysis.

Bioinformatics

DIANA Tools (<http://diana.imis.athena-innovation.gr/DianaTools>) was used for in silico prediction of miR-23a binding sites within the GAS5 transcript. On this basis, it was assumed that binding domains existed within the GAS5 transcript and miR-23a. Additionally, GAS5 mutant transcript was generated according to the binding sites of miR-23a within the GAS5 transcript.

Luciferase reporter assay

MT2A 3'UTR cDNA fragments containing miR-23a binding sites were amplified and sub-cloned into the pGL3 luciferase reporter vector (Promega). The resulting constructs, or control luciferase construct, were co-transfected into cells, together with miR-23a mimics or GAS5 plasmids. After 24 h of transfection, luciferase activity was measured by using the Dual Luciferase Reporter Assay System (Promega).

Luciferase activity results were normalized to those of the control group. All reactions were repeated in triplicate through at least three independent experiments.

Western blot

The protein concentrations of the total cell lysates were measured using the Micro BCA protein assay kit and were separated on SDS-PAGE (10%) and transferred to nitrocellulose membranes. The specific antibody for MT2A was purchased from Abcam. β -actin was used as a control. The membrane was incubated with the secondary antibody for 2 h. Finally, the enhanced chemiluminescence (ECL) kit was used according to the manufacturer's instructions and to visualize the immunoreactive protein bands.

Statistical analysis

Statistical analysis was performed using the SPSS 18.0 software. The values are presented as the mean \pm SD. The data was calculated using Student's t-test or one-way analysis of variance (ANOVA). P values less than 0.05 were considered significant.

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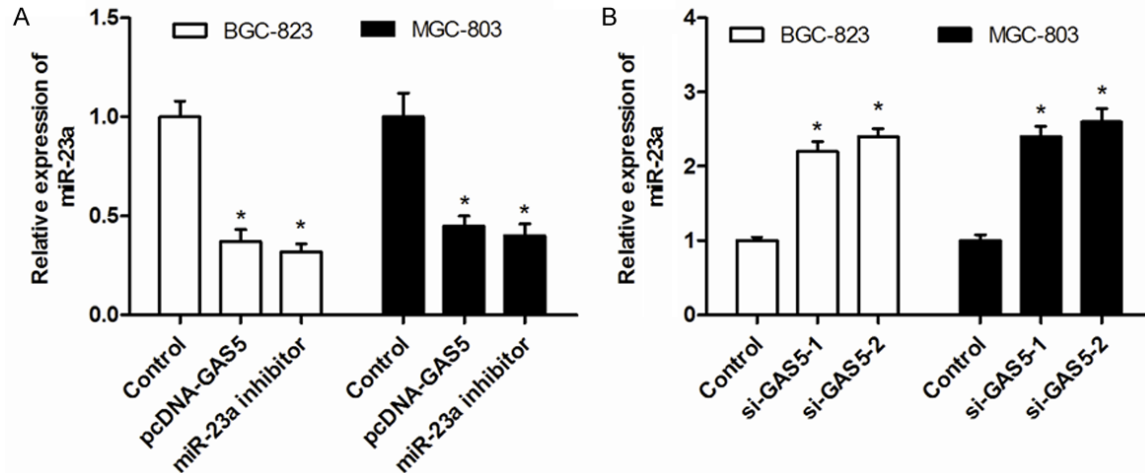


Figure 2. Regulation of GAS5 on miR-23a expression through gain- and loss-of function studies. (A) The expression of miR-23a was downregulated in GAS5 overexpressing GC cells (pcDNA-GAS5) (miR-23a inhibitor serving as a positive control). (B) The expression of miR-23a was upregulated in GAS5 inhibition GC cells. Control: Cells with pcDNA 3.1 (A) or si-NC (B) transfection. *P<0.05.

Results

GAS5 was negative correlated with miR-23a in human gastric cancer tissues

The expression of GAS5 and miR-23a were explored by qRT-PCR analysis in gastric cancer tissues and adjacent non-tumor tissues. qRT-PCR showed that GAS5 levels were lower in GC tissues than in adjacent non-tumor tissues (**Figure 1A**, P<0.05). For miR-23a, our results showed that the expression level of miR-23a was significantly up-regulated in GC tissues compared with adjacent non-tumor tissues (**Figure 1B**, P<0.05). Furthermore, correlation analysis showed that there was a negative correlation between GAS5 and miR-23a expression in GC tissues (**Figure 1C**, $R^2=0.5149$, P<0.05), suggesting the negative regulatory mechanism between GAS5 and miR-23a.

GAS5 regulated miR-23a expression in gastric cancer cells

To further explore the potential relationship between GAS5 and miR-23a, gain- and loss-of function studies were performed in gastric cancer cells. We found that the expression level of miR-23a was decreased in GAS5 overexpressing GC cells, (miR-23a inhibitor transfection serving as the positive control) (**Figure 2A**, P<0.05). Moreover, we showed that inhibition of GAS5 increased miR-23a expression in GC cells (**Figure 2B**, P<0.05). These findings con-

firmed that GAS5 showed negative regulation of miR-23a expression in GC cells, and indicated that GAS5 might function as an inhibitor of miR-23a by sponging miR-23a through their potential interaction.

GAS5 regulated MT2A expression by sponging miR-23a

Bio-informatics analysis for the predicted interaction revealed potential binding domains within GAS5 transcripts and miR-23a (**Figure 3A**). Then, the potential regulation of GAS5 on the expression of MT2A, one validated miR-23a target, was explored in cells with abnormal GAS5 expression. Our results showed that MT2A mRNA was significantly up-regulated in GAS5 overexpressed cells, while the expression levels of MT2A mRNA was down-regulated in the GAS5 inhibited cells (**Figure 3B** and **3D**, P<0.05). Moreover, luciferase reporter assay suggested that GAS5 overexpression inhibited miR-23a induced decrease of MT2A 3'UTR activity, while no obvious change was obtained by further overexpression of GAS5 mutant in miR-23a overexpressing cells (**Figure 3C**, P<0.05). Thus, those data suggested that GAS5 could regulate MT2A expression by sponging miR-23a in GC cells.

MT2A expression was correlated with GAS5 and miR-23a

In order to further explore the correlation between MT2A and GAS5 or miR-23a, MT2A

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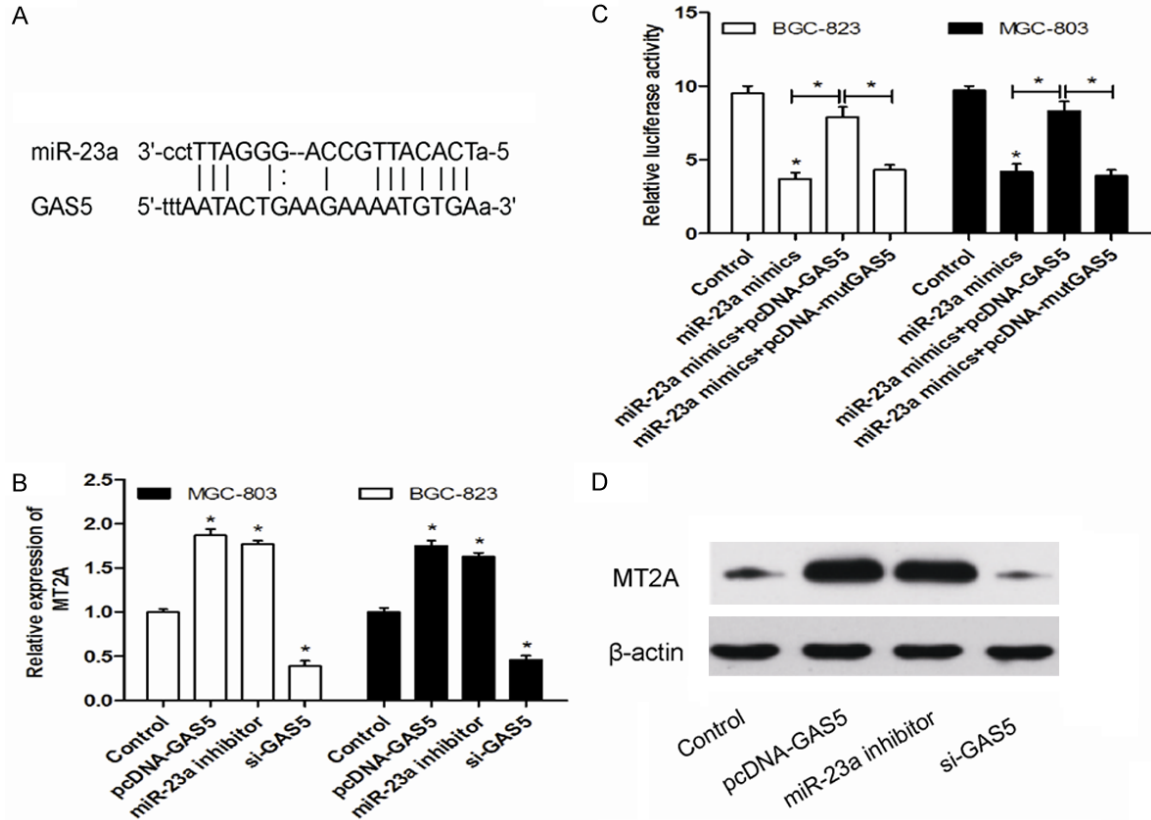


Figure 3. Potential regulation of GAS5 on MT2A expression through miR-23a mediated targeting of MT2A. A. Bioinformatics analysis the combination of GAS5 and miR-23a. B. The expression of MT2A mRNA in GC cells with abnormal GAS5 expression (miR-23a inhibitor act as positive control). C. Luciferase report analysis for the activity of 3'UTR under miR-23a mimic transfection or co-transfection with normal pcDNA-GAS5 plasmid or pcDNA-mutGAS5 plasmid. D. The expression level of MT2A protein with abnormal GAS5 expression. *P<0.05.

expression was detected in gastric cancer tissues and adjacent non-tumor tissues by qRT-PCR. Our results revealed that the expression level of MT2A was decreased in GC tissues compared to adjacent non-tumor tissues (Figure 4A, P<0.05). Further correlation analysis indicated that there is a negative relationship between MT2A and miR-23a (Figure 4B, R²=0.4798, P<0.05), in addition, there was a positive relationship between MT2A and GAS5 (Figure 4C, R²=0.4798, P<0.05). These data supported the role of GAS5 act as a miR-23a sponge in GC pathogenesis.

Discussion

Long non-coding RNA GAS5 was originally identified via subtractive cDNA cloning of genes which are preferentially expressed in growth-arrested cells [21]. Recently studies showed that aberrant expression of GAS5 was discov-

ered in types of tumors and many vital biological processes including tumor cell proliferation, apoptosis, invasion and metastasis were closely associated with GAS5 [22]. Previous study showed that GAS5 was down-regulated in gastric cancer tissues and cells, wherein it might function as a tumor suppressor by decrease gastric cancer cell proliferation and induce apoptosis [15]. However, the underlying mechanism of the tumor suppressive role of GAS5 in gastric cancer was still unclear.

Increasing evidence showed that lncRNA may function as a competitor to endogenous RNA or molecular sponge in regulating the expression and biological functions of miRNA when the expression of lncRNA and miRNA shows an inverse correlation [23]. In the present study we showed that GAS5 expression was down-regulated in GC tissues and showed an inverse correlation with up-regulated miR-23a.

GAS5 regulate GC progression via miR-23a

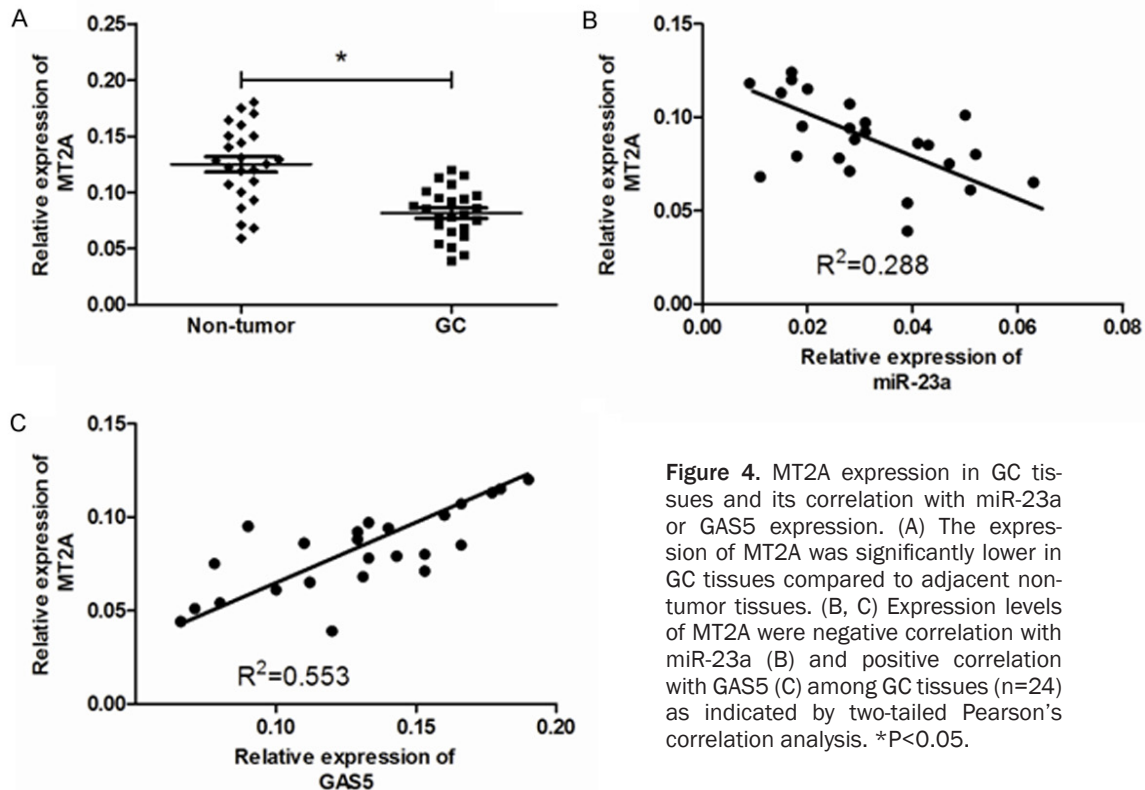


Figure 4. MT2A expression in GC tissues and its correlation with miR-23a or GAS5 expression. (A) The expression of MT2A was significantly lower in GC tissues compared to adjacent non-tumor tissues. (B, C) Expression levels of MT2A were negative correlation with miR-23a (B) and positive correlation with GAS5 (C) among GC tissues (n=24) as indicated by two-tailed Pearson's correlation analysis. *P<0.05.

Moreover, bio-informatics prediction revealed that miR-23a binding domains within GAS5 transcripts, indicating that GAS5 may serve as a miR-23a inhibitor through direct interaction between miR-23a and its binding sites within GAS5 transcripts. As we know, miRNAs can inhibit gene expression at the post-transcriptional level by binding the 3'UTR of their target mRNA [24]. So these lncRNAs serving as miRNA sponges can also regulate the expression and biological functions of miRNA targets [20].

Metallothionein 2A (MT2A), a direct target of miR-23a [18], is a major component of the mucosal barrier in the gastrointestinal tract [25]. Several studies demonstrated a role for MT2A in carcinogenesis, tumor development, chemotherapy resistance and prognosis [26]. Recent study showed that up-regulation of MT2A enhanced chemosensitivity of human gastric cancer cells to docetaxel through attenuating NF- κ B activation [27]. Thus, in order to illustrate the role of GAS5 as a miR-23a sponge, the potential regulation of GAS5 on MT2A expression was also analyzed in gastric cancer cells with abnormal GAS5 expression. Expression analysis and the luciferase reporter assay showed that GAS5 negatively regulated

the inhibition of miR-23a on MT2A expression and also miR-23a binding with MT2A 3'UTR, with the GAS5 mutant transcript not being capable of binding to miR-23a, and thereby exerting little influence on miR-23a induced effects. These results demonstrated a positive regulation of GAS5 on the miR-23a target MT2A. In addition, a strongly down-regulated expression of MT2A was also confirmed in GC tissues and respectively shows positive or negative correlation with that of GAS5 or miR-23a. Therefore, it was concluded that GAS5 may regulate the cell proliferation and apoptosis of GC cells through the regulation of MT2A, partially by sponging miR-23a.

In conclusion, our results have provided strong evidence that GAS5 can function as a competing endogenous RNA to promote MT2A expression by sponging miR-23a and potentially serve as a therapeutic target in gastric cancer.

Disclosure of conflict of interest

None.

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