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miR-107 and miR-25 simultaneously target LATS2 and regulate proliferation and invasion of gastric adenocarcinoma (GAC) cells

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ABSTRACT

Although a series of oncogenes and tumor suppressors were identified in the pathological development of gastric adenocarcinoma (GAC), the underlying molecule mechanism were still not fully understood. The current study explored the expression profile of miR-107 and miR-25 in GAC patients and their downstream regulative network. qRT-PCR analysis was performed to quantify the expression of these two miRNAs in serum samples from both patients and healthy controls. Dual luciferase assay was conducted to verify their putative bindings with LATS2. MTT assay, cell cycle assay and transwell assay were performed to explore how miR-107 and miR-25 regulate proliferation and invasion of gastric cancer cells. Findings of this study demonstrated that total miR-107 or miR-25 expression might be overexpressed in gastric cancer patients and they can simultaneously and synchronically regulate LATS2 expression, thereby affecting gastric cancer cell growth and invasion. Therefore, the miR-25/miR-107-LATS2 axis might play an important role in proliferation and invasion of the gastric cancer cells.

1. Introduction

Gastric cancer (GC) ranked the fourth most common malignancy and the second leading cause of cancer related death across the world [1]. Gastric adenocarcinoma (GAC) accounts for over 90% cases of all GC [1]. Although a series of oncogenes and tumor suppressors were identified in the pathological development of GAC, the underlying molecule mechanism were still not fully understood.

Aberrant expression of microRNAs (miRNAs) is associated with GAC development [2,3]. Some of the miRNAs might play critical regulative roles in cell proliferation, metastasis, differentiation, development and apoptosis [4]. Gastric carcinogenesis involved multistep processes that are related to many environmental and genetic factors [5] and different miRNAs might be involved in different pathological processes. MiR-107 and miR-25 are two miRNAs significantly upregulated in some cases of GAC [5–7]. In fact, the oncogenic roles of miR-25 and miR-107 in gastric cancer were already observed in some previous studies. One recent study reported that miR-25 can promote gastric cancer cell growth and motility by targeting RECK [8]. It can also promote gastric cancer migration, invasion and proliferation by directly targeting transducer of ERBB2 and 1 [9]. High miR-25 expression also indicates poor survival of the GC patients [9]. MiR-107 can promote proliferation of gastric cancer cells by targeting CDK8 [10] and FOXO1 [11]. It can also regulate gastric cancer cell invasion and metastasis by targeting DICER1 [12]. Therefore, these two miRNAs might be two important oncomiRs in GC.

However, a miRNA may have multiple targets, even in one type of cancer. Whether miR-107 and miR-25 have other targets in GAC is not clear. In the current study, we explored the expression pattern of these two miRNAs in GAC patients and firstly reported that miR-107 and miR-25 can simultaneously targeting large tumor suppressor 2 (LATS2) and synchronically modulate its expression, thereby promoting proliferation and invasion of GAC cells.

2. Methods

2.1. Human specimens

All of the subjects were recruited from the cancer center of the Second Affiliated Hospital of Anhui Medical University from January 2013 to Sep 2014 in Anhui province, China. GAC was

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confirmed by histopathological examination of tumor tissues after physical resection. The tumor stages were determined by experienced pathologists without authorship to this study according to the International Union Against Cancer's (UIAC) tumor-node-metastasis (TNM) system. Healthy controls were recruited from blood donation center of the hospital. Participants who had surgical operation, with self-reported previous cancer history or had previous chemo-radiotherapy were excluded. 5 ml of venous blood was collected from each participant at the recruitment. A total of 28 participants (14 healthy controls and 14 GCA cases) were included in this study and their basic features were summarized in Supplementary Table 1.

2.2. Cell culture

Immortalized normal human gastric epithelial cell line GES-1, human GCA cell line BGC-823 and SGC-7901 were obtained from Shanghai Institute of Cell Biology, China Academy of Sciences (Shanghai, China). HEK293T cells and human gastric adenocarcinoma cell line AGS was obtained from ATCC. All cells were cultured in RPMI-1640 medium supplemented with 10% FBS at 37 °C in 5% CO_2.

2.3. Cell transfection

AGS cells and SGC-7901 cells were transfected with 50 nM miR-25 or miR-107 mimics, 100 nM antagomiR-25 or antagomiR-107, co-transfected with 40 nM miR-25 and 40 nM miR-107 mimics, or corresponding negative controls (RiboBio, China) using Lipofectamine RNAiMAX (Invitrogen, USA). AGS and SGC-7901 cells transfected with 50 nM miR-25 or miR-107 mimics were further transfected with pcDNA3.1-LATS2 (without 3′UTR) or pcDNA3.1 empty vector using Lipofectamine 2000 reagent (Invitrogen).

2.4. qRT-PCR analysis of miR-25/107 and LATS2 expression

Total RNAs were extracted from serum and cell samples using the mirVana PARIS Kit (Ambion, USA) according to manufacturer's protocol. The cDNA was reversely transcribed by using TaqMan MicroRNA Reverse Transcription Kit. To determine miR-25 and miR-107 expression levels, qRT-PCR was performed using TaqMan MicroRNA Assays (Applied Biosystems). U6 snRNA served as an internal control.

To assess LATS2 mRNA expression, total RNAs in cells were extracted using Trizol Reagent (Invitrogen, USA) firstly. Then, the first strand cDNA was reversely transcribed by using RevertAid first strand cDNA synthesis kit (Fermentas). LATS2 mRNA level was quantified by qRT-PCR analysis using Syber Green PCR Master Mix (Applied Biosystems, USA) and LATS2 specific primers : forward: 5′-AAGAGCTACTCGCCATACGCCTTT-3′, reverse: 5′-AGCTTTGGCCA TTTCTTGCTCCAG-3'. GAPDH served as internal control. 2-ΔCt value was calculated to indicate relative expression of each transcript in serum samples from patients, while 2-ΔΔCt was used to calculate relative expression in cell samples.

2.5. Cell proliferation assay

24 h after transfection, cells were seeded into 96-well plates at a density of 1×10^3 cells/well in a final volume of 100 μL and further cultured for 24, 48 or 72 h. At indicated time point, MTT (Sigma−Aldrich) (20 μL of 0.5 mg/mL) was added to each well for 4 h at 37 °C. Then, the culture medium was replaced by 150 μL DMSO (Sigma, USA). Absorbance at 490 nm of the solution was measured.

2.6. Cell cycle analysis

48 h after transfection, cells were harvested and fixed in 70% ice-cold ethanol at 4 °C for 24 h. Then, the cells were incubated with 20 μg/ml propidium iodide (PI) (Sigma) for 20 min at room temperature. Cell cycle distribution was determined using flow cytometry (FACSCalibur, BD Biosciences, USA).

2.7. Transwell analysis of cell invasion

Transwell insert chamber coated with Matrigel (BD Biosciences) was used for cell invasion assay. Generally, 1×10^5 cells were suspended in 200 μL serum free RPMI-1640 medium and seeded into the upper chamber of the insert with Matrigel. The lower chamber was filled with RPMI-1640 supplemented with 20% FBS as the chemoattractant. After 24 h incubation at 37°Cin 5% CO_2 in humidified incubator, cells on the top surface of the insert were removed and the cells on the bottom surface were fixed with 4% polyoxymethylene and stained with 0.1% crystal violet for 20 min. Cell counting was performed at 100× magnification under a microscope. Each experiment was performed in triplicate.

2.8. Dual luciferase assay

The binding sites between miR-107/miR-25 and human LATS2 mRNA were predicted in Targetscan 6.2. The wide type and designed mutant LATS2 3′UTR sequence with or without miR-107/25 specific binding sites were chemically synthesized and cloned into pGL3 promoter vector to construct recombinant reporters, namely miR-107-LATS2-wt, miR-107-LATS2-mut, miR-25-LATS2-wt and miR-25-LATS2-mut, respectively. HEK293T cells were co-transfected miR-107-LATS2-wt or miR-25-LATS2-wt and miR-181b mimics. AGS cells were co-transfected miR-107-LATS2-wt and antagomiR-107. SGC-7901 cells were co-transfected miR-25-LATS2-wt and antagomiR-25. 24 h after transfection, luciferase activity was analyzed using the Dual-Luciferase Reporter Assay System (Promega, USA). Firefly luciferase activity was normalized to that of Renilla luciferase.

2.9. Statistical analysis

Data were represented as mean ± SD from at least three repeats. Group difference was assessed using Student's t-test. $P < 0.05$ was considered statistically significant. *, **, and *** donate significance at 0.05, 0.01 and 0.001 level respectively.

3. Results

3.1. Total miR-107 and miR-25 expression is significantly increased in GAC patients

To explore the expression profile of miR-107 and miR-25 in GAC patients and healthy controls, serum samples from 28 participants (GAC = 14 and healthy control = 14) were used for qRT-PCR analysis. Although the average miR-107 expression in the GAC patients is generally significantly higher than in healthy control, but this upregulation is not consistent in all patients. Five selected patients have average miR-107 expression even lower than that of control group (Fig. 1A and C). Similar trend was observed in miR-25 comparison. Its expression is generally higher in GAC patients than in healthy controls, but the expression in six selected patients was similar to that of controls (Fig. 1B and D). Interestingly, we observed that the five patients with low miR-107 level had had high miR-25 expression, while six patients with low miR-25 level had high miR-107 expression respectively. Therefore, we quantified the total

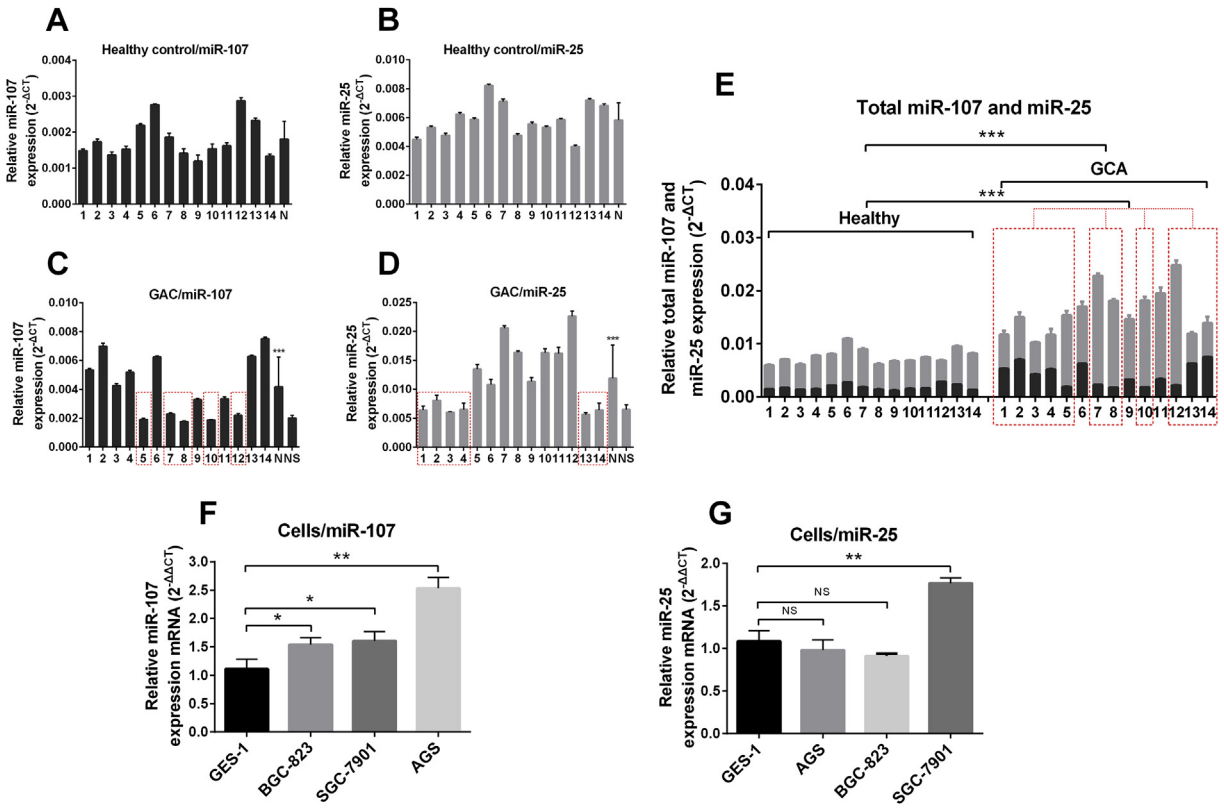

Fig. 1. Total miR-107 and miR-25 expression is significantly increased in GAC patients. (A and B) qRT-PCR analysis of miR-107 (A) and miR-25 (B) expression in serum of 14 healthy participants. (C and D) qRT-PCR analysis of miR-107 (C) and miR-25 (D) expression in serum of 14 GAC cases. Bars in red frame were patients with relatively low miR-107 or low miR-25 expression. (E) Comparison of total miR-107 and miR-205 expression in GAC patients and healthy controls. Bars in red frame were patients with relatively low miR-107 or low miR-25 expression. (F and G) qRT-PCR analysis of relative miR-107 (F) and miR-25 (G) expression in GES-1, GBC-823, SGC-7901 and AGS cells. N: group average. NS: average of selected samples in red frame. In figure C and D, "***" indicates comparison results of group average between healthy and cancer groups. Values are the average of triple determinations with the S.D. indicated by error bars. *P < 0.05, **P < 0.01 and ***P < 0.001. NS: not significant. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

expression of miR-107 and miR-25 expression in the patients. As expected, we found that the total expression of these two miRNAs was significantly higher in GAC patients than in healthy controls (Fig. 1E). More importantly, the total miR-107 and miR-25 expression in the selected low miR-107 and low miR-25 patients was also significantly higher than that in healthy controls (Fig. 1E), suggesting these two miRNAs may have synergetic expression in GAC. Then, we further studied miR-107 and miR-25 expression in normal human gastric epithelial cell line GES-1 and human GAC cell line BGC-823, SGC-7901 and AGS. qRT-PCR analysis showed that AGS and SGC-7901 cells had the highest miR-107 and miR-25 expression respectively (Fig. 1F and G).

3.2. Both miR-107 and miR-25 can promote proliferation and invasion of GAC cells

To explore the possible role of miR-107 and miR-25 in gastric cancer, AGS cells with relatively high miR-107 expression and SGC-7901 cells with relatively high miR-25 expression were firstly transfected with miR-107/25 mimics or antagomiR-107/25 respectively. MTT assay showed that both miR-107 and miR-25 overexpression induced a significant increase in cell proliferation in both AGS and SGC-7901 cells (Fig. 2B and D). AGS cells with miR-107 knockdown and SGC-7901 cells with miR-25 knockdown had significantly inhibited cell growth. SGC-7901 cells with miR-107 knockdown also had inhibited cell growth. But this trend was not observed in AGS cells with miR-25 knockdown (Fig. 2A and C). Then, we subsequently studied how miR-107 and miR-25 overexpression influence cell cycle progression. In both AGS and SGC-7901 cells, miR-107 and miR-25 overexpression can both induce decreased proportion of cells in the G1/G0 phase and increased S phase accumulation (Fig. 2E and F). Therefore, these two miRNAs might regulate cancer cell growth through enhancing cell cycle progression at G1/S transition. Transwell assay was used to assess how miR-107 and miR-25 overexpression influence cell invasion. miR-107 and miR-25 overexpression both promoted cell invasion in both AGS and SGC-7901 cells (Fig. 2G and H). These results suggest that both miR-107 and miR-25 can promote proliferation and invasion of gastric cancer cells.

3.3. Both miR-107 and miR-25 can target LATS2 and regulate its expression

Since miR-107 and miR-25 has similar effect in promoting gastric cancer cell growth and invasion, we further explored their downstream network. Through prediction in bioinformatics databases, we identified LATS2 as a common target of these two miRNAs (Fig. 3A and B). Then we designed mutant sequence without the putative bindings and construct corresponding luciferase reporters, namely miR-107-LATS2-wt, miR-107-LATS2-mut, miR-25-LATS2-wt and miR-25-LATS2-mut, respectively. In dual luciferase reporter assay, both miR-107 and miR-25 mimics could only significantly inhibit transcriptional activity of miR-107-LATS2-wt and miR-25-LATS2-wt, but had no effect on reporters with

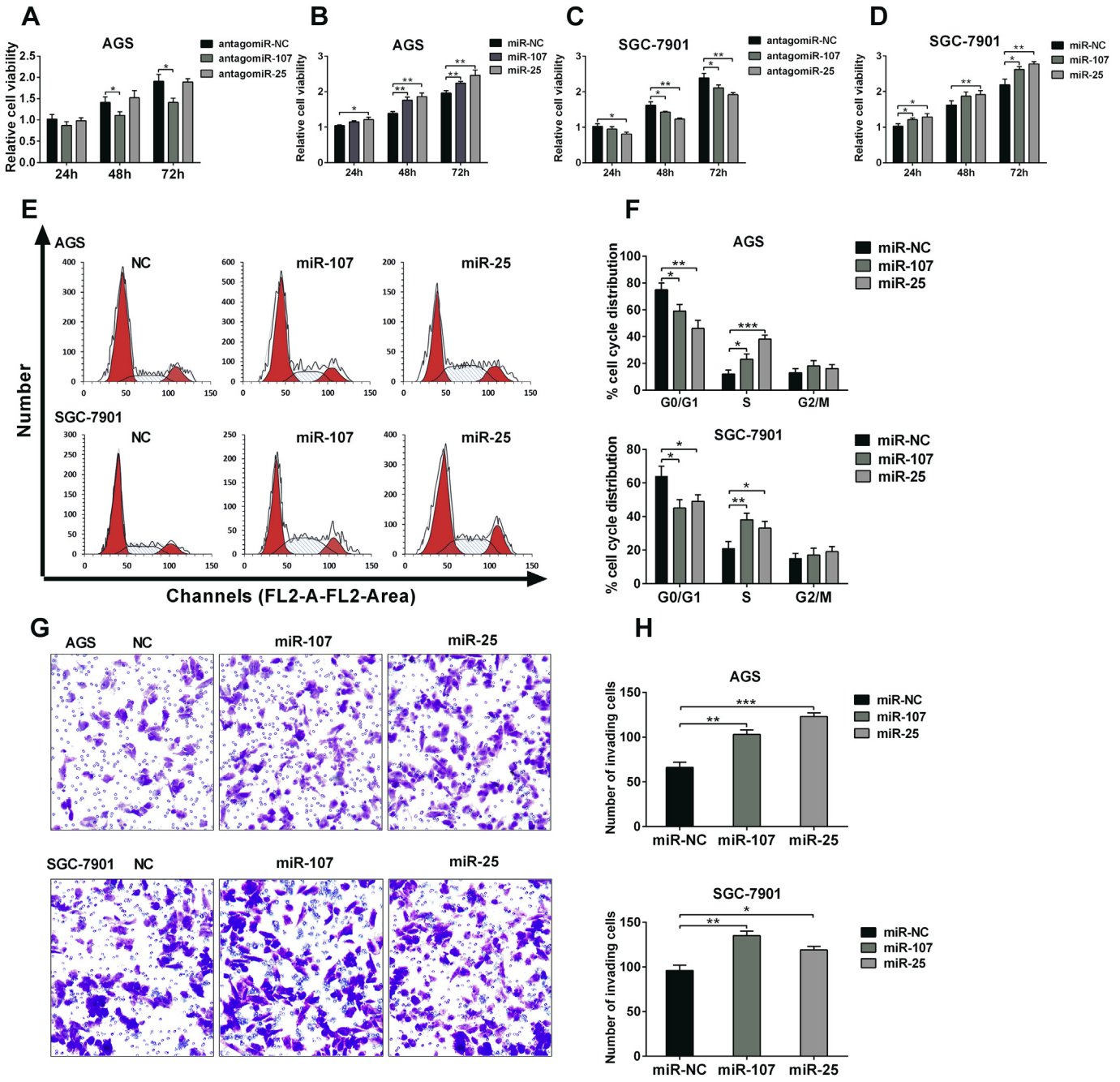

Fig. 2. Both miR-107 and miR-25 can promote proliferation and invasion of GAC cells. (A–D) MTT assay of cell viability of AGS (A and B) and SGC-7901 (C and D) cells transfected 50 nM antagomiR-107 or 50 nM antagomiR-25 (A and C) or transfected with 50 nM miR-107 mimics or 50 nM miR-25 mimics (B and D) at 24, 48 and 72 h measurement after transfection. (E) Representative images of flow cytometry analysis of cell cycle distribution of AGS and SGC-7901 cells after transfection of 50 nM miR-107 mimics or 50 nM miR-25 mimics. (F) Quantification of proportion of cells in different cell phases showed figure E. (G) Representative images of transwell assay of cell invasion of AGS and SGC-7901 cells after transfection of 50 nM miR-107 mimics or 50 nM miR-25 mimics. (H) Quantification of invading cells showed figure G. Values are the average of triple determinations with the S.D. indicated by error bars. *P < 0.05, **P < 0.01 and ***P < 0.001.

mutant sequence (Fig. 3C and D). In AGS cells, knockdown of endogenous miR-107 could significantly promote transcriptional activity of miR-107-LATS2-wt but not miR-107-LATS2-mut (Fig. 3E). In SGC-7901 cells, knockdown of endogenous miR-25 could significantly promote transcriptional activity of miR-25-LATS2-wt but not miR-25-LATS2-mut (Fig. 3F). Then, we further detected how these two miRNAs affect LATS2 level at both mRNA and protein level. qRT-PCR analysis showed that miR-107 and miR-25 overexpression alone could significantly decrease LATS2 mRNA level in both AGS and SGC-7901 cells (Fig. 3G and H). Overexpression miR-107 and miR-25 in combination could achieve more evident inhibiting effect than any one alone in both AGS and SGC-7901 cells (Fig. 3G and H). Knockdown of miR-107 and miR-25 increased LATS2 in AGS and SGC-7901 cells respectively (Fig. 3G and H). Western blot analysis also confirmed these changes at protein level (Fig. 3G and H). These results suggest that both miR-107 and miR-25 can directly target LATS2 and modulate its expression.

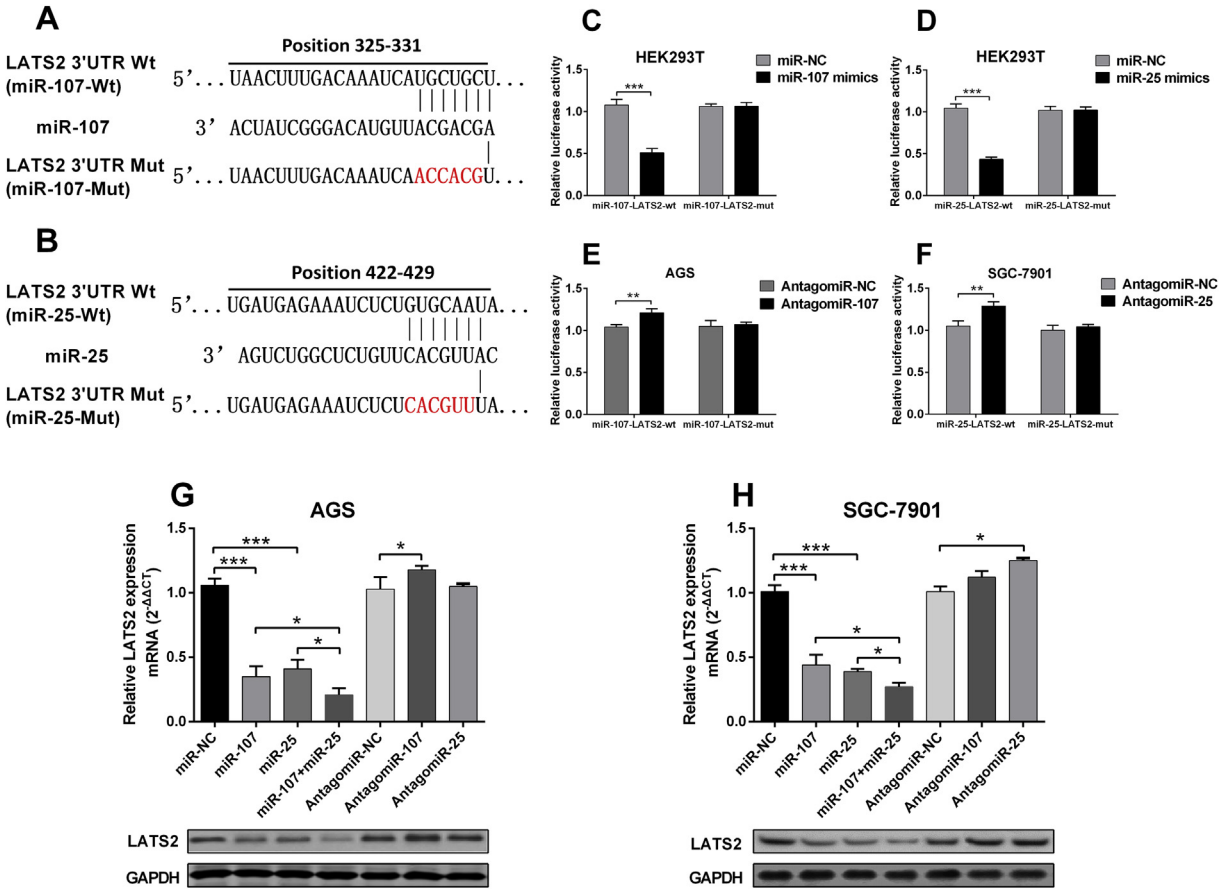

Fig. 3. Both miR-107 and miR-25 can target LATS2 and regulate its expression. (A and B) Predicted binding and corresponding mutant sequence between LATS2 3′UTR and miR-107 (A) and miR-25 (B). (C and D) Dual luciferase reporter assay of HEK293T cells co-transfected with miR-107-LATS2-wt/mut and miR-107 mimics (C) or co-transfected with miR-25-LATS2-wt/mut and miR-25 mimics (D). (E and F) Dual luciferase reporter assay of AGS cells co-transfected with miR-107-LATS2-wt/mut and miR-107 mimics (E) or SGC-7901 cells co-transfected with miR-25-LATS2-wt/mut and miR-25 mimics (F). (G and H) qRT-PCR and western blot analysis of LATS2 expression at mRNA and protein level in AGS (G) and SGC-7901 (H) cells transfected with 50 nM miR-107, 50 nM miR-25, 40 nM miR-25 and 40 nM miR-107 mimics, 100 nM antagomiR-25 or 100 nM antagomiR-107. Values are the average of triple determinations with the S.D. indicated by error bars. *P < 0.05, **P < 0.01 and ***P < 0.001.

3.4. miR-107 and miR-25 regulate proliferation and invasion of GAC cells though LATS2

Since LATS2 is a common direct target of miR-107 and miR-25, we further explored its function in GAC cells. Both AGS and SGC-7901 cells transfected with pcDNA3.1-LATS2 (without LATS2-3′UTR) has significantly increased LATS2 expression and this upregulation was not interfered by miR-107 or miR-25 (Fig. 4A and B). In both AGS cells, LATS2 overexpression could reverse miR-107 and miR-25 induced higher cell proliferation (Fig. 4C and D). In addition, enforced LATS2 expression also abrogated miR-107 and miR-25 induced cell cycle progression at G1/S transition (Fig. 4E and F). Furthermore, restoration of LATS2 expression also counteracted miR-107 and miR-25 induced higher cell invasion (Fig. 4G and H). These results suggest that both miR-107 and miR-25 can regulate proliferation and invasion of GAC cells though LATS2.

4. Discussion

Based on available evidence, upregulation of miR-107 and miR-25 in gastric cancer is closely associated with cancer growth, invasion and metastasis in gastric cancer. miR-107 can simultaneously target CDK8, FOXO1, DICER1 [10–12] and miR-25 can simultaneously target RECK, ERBB2 and 1 [8,9] and thereby affecting proliferation, migration, invasion and/or metastasis pf gastric cancer. Although miR-107 and miR-25 are both viewed as oncomiR in GC, their upregulation was not consistently reported in previous studies [6]. In this study, we also observed that miR-107 and miR-25 were not consistently upregulated in all GAC patients recruited. However, we observed an interesting phenomenon that these two miRNAs might be overexpressed in terms of total expression in the GAC patients. In fact, according to previous studies, upregulation of miR-107 and miR-25 can both enhance proliferation and invasion of human gastric cancer cells [8,9,11,12]. In the current study, we also confirmed that miR-107 and miR-25 overexpression promoted the growth and invasion of gastric cancer cells and enhanced cell cycle transition at G1/S phase. This triggered our interest to explore whether they share some common regulative routes in GAC.

Based on prediction in online informatics databases, we identify LATS2, as a common target of miR-107 and miR-25. LATS2 is a member of LATS tumor suppressor family [13]. LATS2 regulates diverse cellular processes including cell proliferation, apoptosis, angiogenesis, migration and invasion [14,15]. It is also a negative cell cycle regulator that controls G1/S and/or G2/M transition by promoting apoptosis [15,16]. Therefore, LATS2 downregulation may provide a favorable environment for cancer cell growth [17]. It is downregulated in several cancer types, including lung cancer [18], breast cancer [19] and ovarian cancer [20]. In gastric cancer, LATS2 downregulation is also observed and is related to cell cycle change

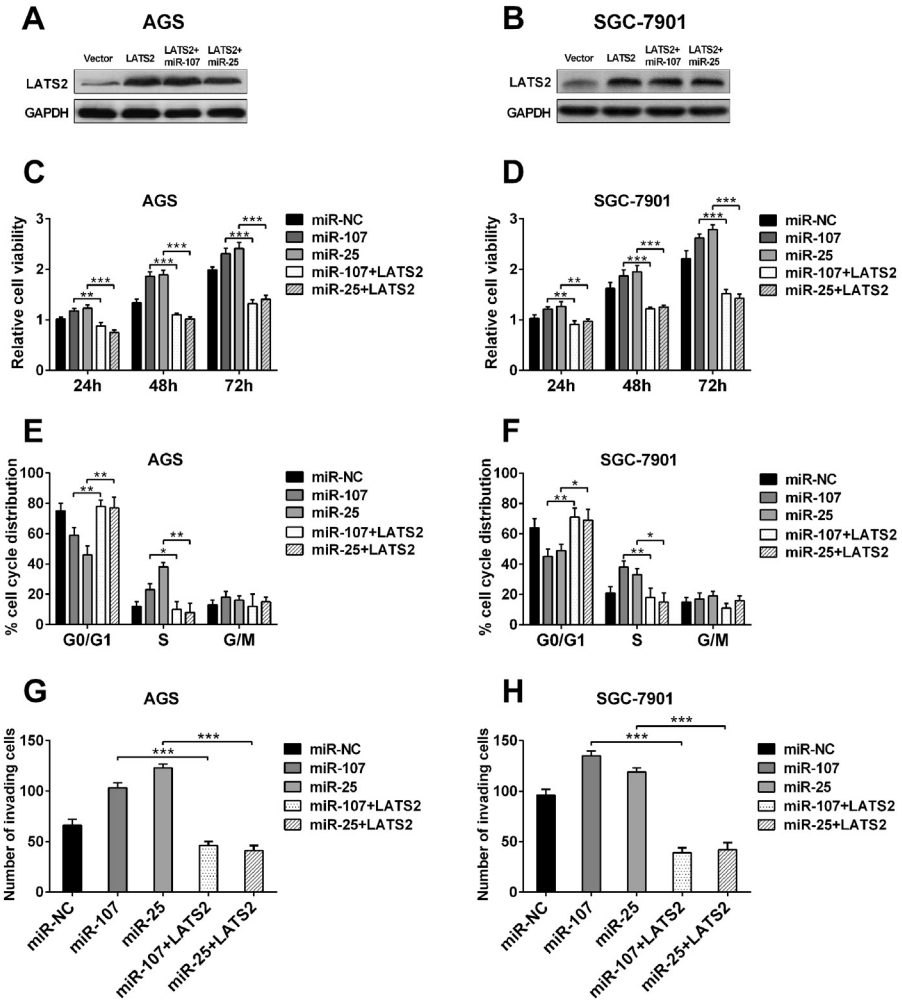

Fig. 4. miR-107 and miR-25 regulate proliferation and invasion of GAC cells though LATS2. (A and B) Western blot analysis of AGS (A) and SGC-7901 (B) cells transfected with LATS2 pcDNA3.1 vectors, or co-transfected with LATS2 pcDNA3.1 and miR-107 mimics or miR-25 mimics. (C and D) MTT assay of cell viability of AGS (C) and SGC-7901 (D) cells co-transfected with LATS2 pcDNA3.1 and miR-107 mimics or miR-25 mimics at 24, 48 and 72 h measurement after transfection. (E and F) Quantification of flow cytometry analysis of cell cycle distribution of AGS (E) and SGC-7901 (F) cells co-transfected with LATS2 pcDNA3.1 and miR-107 mimics or miR-25 mimics. (G and H) Quantification of transwell assay of invading AGS (G) and SGC-7901 (H) cells co-transfected with LATS2 pcDNA3.1 and miR-107 mimics or miR-25 mimics. Values are the average of triple determinations with the S.D. indicated by error bars. *P < 0.05, **P < 0.01 and ***P < 0.001.

and decreased apoptosis of cancer cells [21]. However, its upstream regulative network in gastric cancer is still not quite clear. One previous study found miR-372 is an upstream regulator of LATS2 and regulate cell cycle and apoptosis through this protein [21]. In fact, LATS has an important role in cell cycle regulation and is regulated by multiple miRNAs in different cancers, such as miR-195 in human hepatocellular carcinoma [22], miR-25 and miR-181 in ovarian cancer [20,23] and miR-93 in breast cancer [24]. Since LATS2 is already confirmed a target of miR-25, we firstly verified this regulation in gastric cancer cell lines. Then, considering the synchronic expression of miR-25 and miR-107 in gastric cancer and the putative bindings between miR-107 and LATS2, we further verified this binding in gastric cancer cells. Dual luciferase assay confirmed the bindings and subsequent qRT-PCR and western blot analysis showed that miR-107 can also regulate LATS2 expression. More importantly, overexpression miR-25 and miR-107 simultaneously in AGS and SGC-7901 cells had stronger effect in inhibiting LATS2 expression than miR-107 or miR-25 alone. These results demonstrated that miR-25 and miR-107 can simultaneously and synchronically regulate LATS2 expression in gastric cancer cells. Then, we enforced LATS2 expression in miR-107 or miR-25 overexpressed cancer cells and found LATS2 overexpression largely abrogated miR-107 and miR-25 induced cell growth and invasion. Therefore, these results confirmed that miR-25/miR-107-LATS2 axis might play an important role in proliferation and invasion of the gastric cancer cells.

In conclusion, total miR-107 or miR-25 expression might be overexpressed in gastric cancer patients and they simultaneously and synchronically regulate LATS2 expression, thereby affecting cell growth and invasion. However, one major limitation of this study is the small number GAC patients recruited. Future large epidemic study is required to confirm the general expression pattern of miR-107 and miR-25 in GAC patients.

Conflict of interest

None.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.03.110.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.110.

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