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LncRNA RUNX1-IT1 is downregulated in gastric cancer and suppresses the maturation of miR-20a by binding to its precursor

Lei Bao[1,2*], Boxiang Du[3*], Yunhu Guo[2], Huaguo Zhang[2] and Zhongqi Mao[4]

[1]The First Affiliated Hospital of Soochow University, Suzhou, [2]Department of General Surgery, Huai'an Fifth People's Hospital, Huai'an, [3]Department of Anesthesiology, The Second Affiliated Hospital of Nantong University, Nantong and [4]Department of General Surgery, The First Affiliated Hospital of Soochow University, Suzhou, China

*Lei Bao and Boxiang Du contributed equally to this work

Summary. Background. RUNX1-IT1 has been characterized as a tumor suppressive long non-coding RNA (lncRNA) in several types of cancer but not gastric cancer (GC). This study aimed to explore the role of RUNX1-IT1 in GC.

Methods. The expression of RUNX1-IT1, microRNA (miR)-20a precursor and mature miR-20a in GC and healthy tissues donated by GC patients (n=62) were measured by RT-qPCR. Correlation analysis was performed by linear regression. The expression of mature miR-20a and miR-20a precursor in cells with overexpression of RUNX1-IT1 was also determined by RT-qPCR. Cell invasion and migration were evaluated by Transwell assays.

Results. RUNX1-IT1 was downregulated in GC. Across GC tissues, RUNX1-IT1 and mature miR-20a were inversely correlated. However, RUNX1-IT1 and miR-20a precursor were not closely correlated. RUNX1-IT1 and miR-20a precursor were predicted to interact with each other, and overexpression of RUNX1-IT1 in GC cells decreased the expression levels of mature miR-20a. Transwell assay showed that the enhancing effect of miR-20a on cell invasion and migration was reduced by overexpression of RUNX1-IT1.

Conclusions. RUNX1-IT1 may suppress the GC cell movement by inhibiting the maturation of miR-20a.

Key words: Gastric cancer, RUNX1-IT1, miR-20a, Maturation

Corresponding Author: Zhongqi Mao, Department of General Surgery, the First Affiliated Hospital of Soochow University, No.899 Pinghai Road, Suzhou City, Jiangsu Province, 215000, PR. China. e-mail: zhongqimaoworse@163.com

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Introduction

Gastric cancer is a common type of malignancy for both mortality and incidence and is a heavy burden on global health (Thrift and El-Serag, 2020). In 2018, GC affected a total of 1,033,701 new cases, which were 5.7% of all cancer cases (Bray et al., 2018). Due to its aggressive nature, GC accounted for 8.2% (782,685) of all cancer deaths in the same year (Bray et al., 2018). Although about 70% of local GC patients can survive 5 years (Hallowell et al., 2019), long-distant metastasis to distant sites, such as lung, brain and bone, is frequently observed, leading to a 5-year survival rate lower than 30% (Orditura et al., 2014; Van Cutsem et al., 2016). Therefore, the treatment of GC still requires novel therapeutic approaches.

Previous studies have identified the main causes of GC, including infection with Helicobacter pylori, smoking and long-term stomach inflammation (Compare et al., 2010; de Martel et al., 2013). Various molecular factors have been found to be involved in the tumorigenesis and progression of GC (Wadhwa et al., 2013). Certain molecular factors are potential targets for the development of targeted therapy due to their functions in regulating gene expression and potentials in the treatment of cancers (Kanat et al., 2015; Lee and Oh, 2016). MicroRNAs (miRNAs) and long non-coding RNAs (lncRNAs) are RNA transcripts that exert their functions by affecting the expression of targeted genes (Farazi et al., 2011; Yang et al., 2014). Therefore, they have great potentials in the development of targeted therapies. LncRNA RUNX1-IT1 suppresses colorectal cancer and hepatocellular carcinoma (Shi et al., 2019; Yan et al., 2019; Sun et al., 2020), while its role in GC is unknown. Our preliminary sequencing data revealed altered expression of RUNX1-IT1 in GC and its close correlation with mature miR-20a (Li et al., 2013). Therefore, this study was carried out to explore the

interaction between RUNX1-IT1 and miR-20a in GC.

Materials and methods

Research subjects

From May 2018 to March 2020, 62 GC patients (newly diagnosed cases; 30 cases of carcinoma and 32 cases of adenocarcinoma; 38 males and 24 females; mean age 52.5±5.7 years old) were enrolled at the First Affiliated Hospital of Soochow University. No therapies for any clinical disorders were performed on these patients within 100 days before admission. Patients with other clinical disorders were excluded from this study. Based on AJCC staging criteria, there were 30 and 32 cases at stage I/II or III/IV, respectively. This study was approved by the Ethics Committee of the First Affiliated Hospital of Soochow University. All the experimental procedures in this study complied with the Declaration of Helsinki. All patients signed the written informed consent.

Gastric tissues and cells

Some patients received surgical resection of the primary tumors, which were dissected to prepare paired GC and non-tumor samples. Prior to therapy, fine needle aspiration was performed to obtain paired tissues from patients who were not appropriate for surgery.

Two human GC cell lines SNU-1 (carcinoma) and AGS (adenocarcinoma) (ATCC, USA) were used. A mixture containing 90% RPMI-1640 medium (Cat #R8758, Sigma-Aldrich) and 10% FBS (Cat#F2442, Sigma-Aldrich) was used for cell culture, which was performed in an incubator with humidity, temperature and CO_2 set to 95%, 37°C and 5%, respectively. Cells were collected when about 80% confluence was reached.

Transient transfections

RUNX1-IT1 or miR-20a was overexpressed in cells by transfecting RUNX1-IT1 vector (pcDNA3.1) or miR-20a mimic into cells. Lipofectamine 2000 (Cat # 11668030, Thermo Fisher Scientific) was used for all transfections. Control (untransfected cells, C) and NC (empty vector- or miRNA NC-transfected cells) experiments were also included. Briefly, vector or miRNA mimic was mixed with Lipofectamine 2000, followed by the addition of cells. After incubation at 37°C, cells were washed with fresh medium. In each

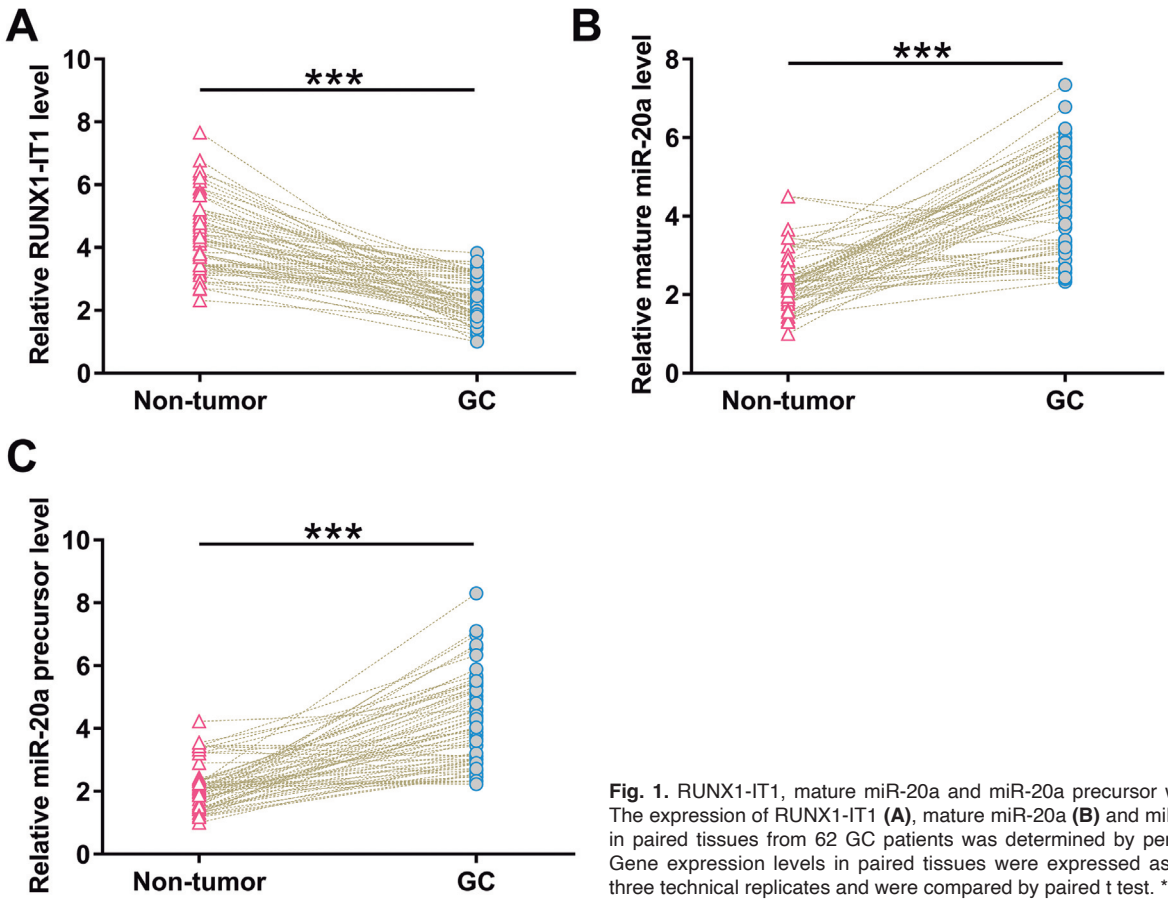

Fig. 1. RUNX1-IT1, mature miR-20a and miR-20a precursor were altered in GC. The expression of RUNX1-IT1 **(A)**, mature miR-20a **(B)** and miR-20a precursor **(C)** in paired tissues from 62 GC patients was determined by performed RT-qPCRs. Gene expression levels in paired tissues were expressed as average values of three technical replicates and were compared by paired t test. ***, $p<0.001$.

transfection, $5x10^6$ cells were transfected with either 10 mM vector or 50 mM miRNA.

Prediction of RNA interaction

The online program IntaRNA 2.0 was used to analyze the potential interaction between RUNX1-IT1 and miR-20a. In this prediction, the long sequence was RUNX1-IT1 and short sequence was miR-20a. Other parameters were all default.

RNA preparations

RNAzol (Cat # R4533, Sigma-Aldrich) was used to isolate total RNAs from paired tissues (100 mg) and SNU-1 and AGS cells ($1x10^6$ cells), followed by incubation with DNase I (Cat # D5025, Sigma-Aldrich) at 37°C for 2h to digest genomic DNA. To detect RNA purity, the OD 260/280 ratio of all RNA samples was determined.

RT-qPCR

Reverse transcriptions (RTs) were performed using the SSRT IV system (Cat #18091050, Thermo Fisher Scientific) to prepare cDNA samples. To determine the expression of RUNX1-IT1, miR-20a precursor and mature miR-20a, qPCRs were performed using the SYBR™ Green PCR Master Mix (Cat #4309155, Thermo Fisher Scientific) with 18S rRNA or U6 as the internal control. The $2^{-\Delta\Delta CT}$ method was used to analyze Ct values. Primer sequences were: 5'-AGAGTCCGA ACGAGAAACC-3' (forward) and 5'-GTGGTATCT GAGCAATGCAA-3' (reverse) for RUNX1-IT1; 5'-AAACGGCTACCACATCCAA-3' (forward) and 5'-TCGCGGAAGGATTTAAAGT-3' (reverse) for human 18S rRNA; 5'-CTCGCTTCGGCAGCACA-3' (forward) and 5'-AACGCTTCACGAATTTGCGT-3' (reverse) for U6; 5'-GTAGCACTAAAGTGCTTA-3' (forward) and 5'-GCAGTACTTTAAGTGCTC-3' (reverse) for miR-20 precursor; 5'-UAAAGTGCTTATAGTGCAG-3' (forward) and oligo d (T) for mature miR-20.

Transwell assays

SNU-1 and AGS cells were used to prepare cell suspensions with cell density set to 300 cells per 1 µl. About 10 µl cell suspension was added to the upper chamber (8 µm, Cat #ab235697, Abcam). The lower chamber was added with 20% FBS to induce cell movement. Matrigel (Cat #354230, Corning)-coated membranes were used in invasion assay. Cell culture was then performed for 12h. After that, membranes were fixed and stained with 1% crystal violet (Cat #548-62-9, Sigma-Aldrich), followed by image analysis under a light microscope.

Statistical analysis

Paired t test was used to compare data of paired tissues. Multiple transfection groups (mean ±SD values) were compared by ANOVA Tukey's test. Linear regression was used for correlation analysis. $p<0.05$ was considered as statistically significant.

Results

The expression of RUNX1-IT1 and miR-20a in GC

The expression of RUNX1-IT1 and miR-20a (both precursor and mature miRNA) in paired tissues donated by the 62 GC patients was detected by RT-qPCRs. It showed that RUNX1-IT1 was significantly down-regulated in GC (Fig. 1A, $p<0.001$). Mature miR-20a (Fig. 1B, $p<0.001$) and miR-20a precursor (Fig. 1C, $p<0.001$) were both highly upregulated in GC tissues.

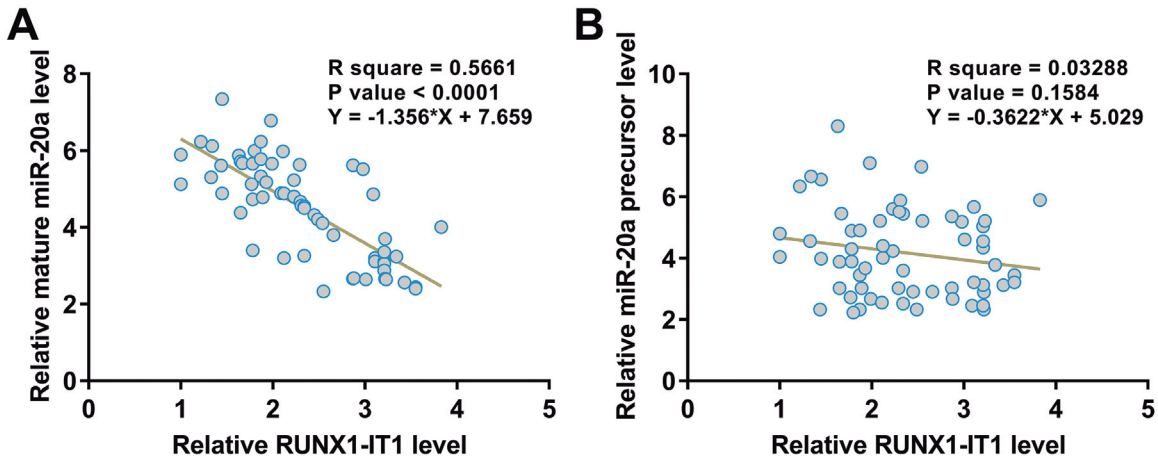

Fig. 2. RUNX1-IT1 was inversely correlated with mature miR-20a, but not miR-20a precursor. The correlations between RUNX1-IT1 and mature miR-20a **(A)** or miR-20a precursor **(B)** were analyzed by linear regression.

These results revealed that the transcription of both RUNX1-IT1 and miR-20a was promoted in GC.

Correlations between RUNX1-IT1 and mature or precursor miR-20a

The correlation of the expression of RUNX1-IT1 with mature (Fig. 2A) or precursor (Fig. 2B) miR-20a was analyzed by linear regression. It showed that RUNX1-IT1 and miR-20a were inversely and significantly correlated across GC tissues at mature level. In contrast, RUNX1-IT1 was not correlated with miR-20a at precursor level.

The role of RUNX1-IT1 in regulating the maturation of miR-20a

The RNA-RNA interaction prediction using IntaRNA 2.0 revealed that RUNX1-IT1 and miR-20a precursor may bind to each other (Fig. 3A, $p<0.05$). RUNX1-IT1 or miR-20a was overexpressed in SNU-1 and AGS cells with RUNX1-IT1 expression vector or miR-20a mimic (Fig. 3B, $p<0.05$). In addition, transfection of RUNX1-IT1 expression vector resulted in downregulation of mature miR-20a (Fig. 3C, $p<0.05$), but not miR-20a precursor (Fig. 3D). In contrast, transfection of miR-20a mimic did not affect the

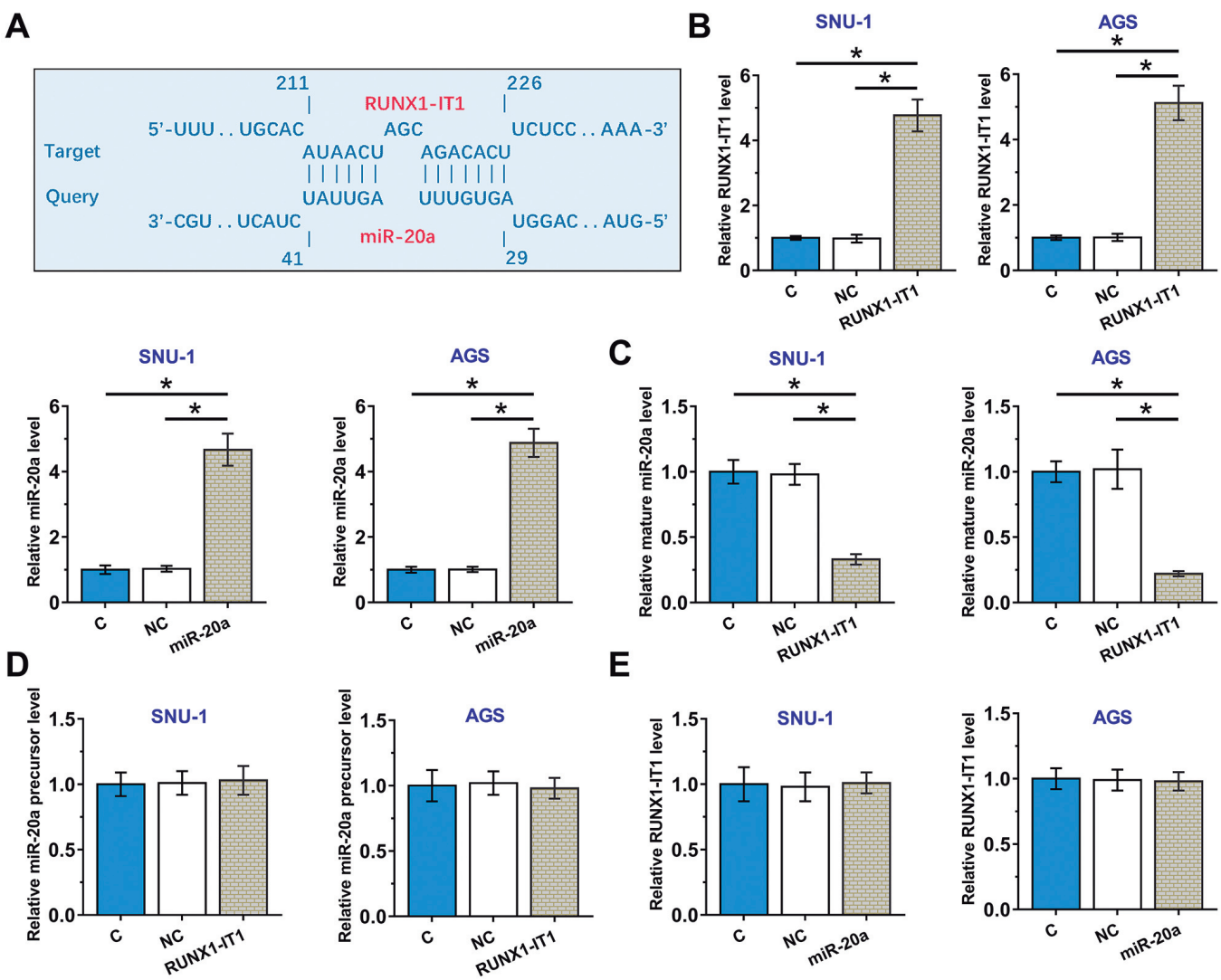

Fig. 3. RUNX1-IT1 may bind miR-20a precursor to suppress the production of mature miR-20a. The interaction between RUNX1-IT1 and miR-20a precursor was predicted by IntaRNA 2.0 **(A)**. SNU-1 and AGS cells were transfected with RUNX1-IT1 expression vector or miR-20a mimic, followed by performing RT-qPCR to confirm the overexpression of RUNX1-IT1 and miR-20a **(B)**. The effects of RUNX1-IT1 expression vector transfection on the expression of mature miR-20a **(C)** and miR-20a precursor **(D)**, and the effects of miR-20a mimic transfection on RUNX1-IT1 (E) were also explored by RT-qPCR. Data of multiple transfection groups were expressed as mean±SD values of three independent replicates. *, $p<0.05$.

expression of RUNX1-IT1 (Fig. 3E).

Overexpression of RUNX1-IT1 inhibited the invasion and migration of SNU-1 and AGS cells through miR-20a

Transwell assays were performed to study cell invasion (Fig. 4A) and migration (Fig. 4B) of SNU-1 and AGS cells. Overexpression of RUNX1-IT1 resulted in decreased cell movement, while overexpression of miR-20a led to increased cell movement. Moreover, the enhancing effect of miR-20a on cell movement was reduced by overexpression of RUNX1-IT1 ($p<0.05$).

Discussion

This study investigated the expression pattern of RUNX1-IT1 and miR-20a in GC and the potential crosstalk between them. The expression of RUNX1-IT1 was elevated in GC and it may prevent the production of mature miR-20a to inhibit the movement of cancer cells.

Previous studies have shown that RUNX1-IT1 plays a tumor suppressive role in colorectal cancer and hepatocellular carcinoma (Shi et al., 2019; Yan et al., 2019; Sun et al., 2020). In colorectal cancer, RUNX1-IT1 is downregulated, and overexpression of RUNX1-IT1 resulted in suppressed oncogenic features (Shi et al., 2019; Yan et al., 2019). In hepatocellular carcinoma, RUNX1-IT1 is also downregulated and can target MAPK pathways to inhibit cell growth and accelerate cell apoptosis (Yan et al., 2019). In another study, RUNX1-IT1 was found to be downregulated in hepatocellular carcinoma by regulation of hypoxia-driven histone deacetylase 3 (Sun et al., 2020). In this study we observed the downregulation of RUNX1-IT1 in GC. Our results demonstrated that overexpression of RUNX1-IT1 led to decreased invasion and migration of both carcinoma and adenocarcinoma cells. Therefore, RUNX1-IT1 may play a tumor suppressive role in both subtypes of GC by suppressing cancer cell invasion and migration.

MiR-20a has been reported to be an oncogenic miRNA in many types of cancer including GC. It was reported that miR-20a is downregulated in GC and can target early growth response to promote cancer cell invasion and migration (Li et al., 2013). Moreover, miR-20a targeted NFKBIB to induce the resistance to cisplatin in cancer cells (Du et al., 2016). In this study we confirmed that miR-20a precursor and mature miR-

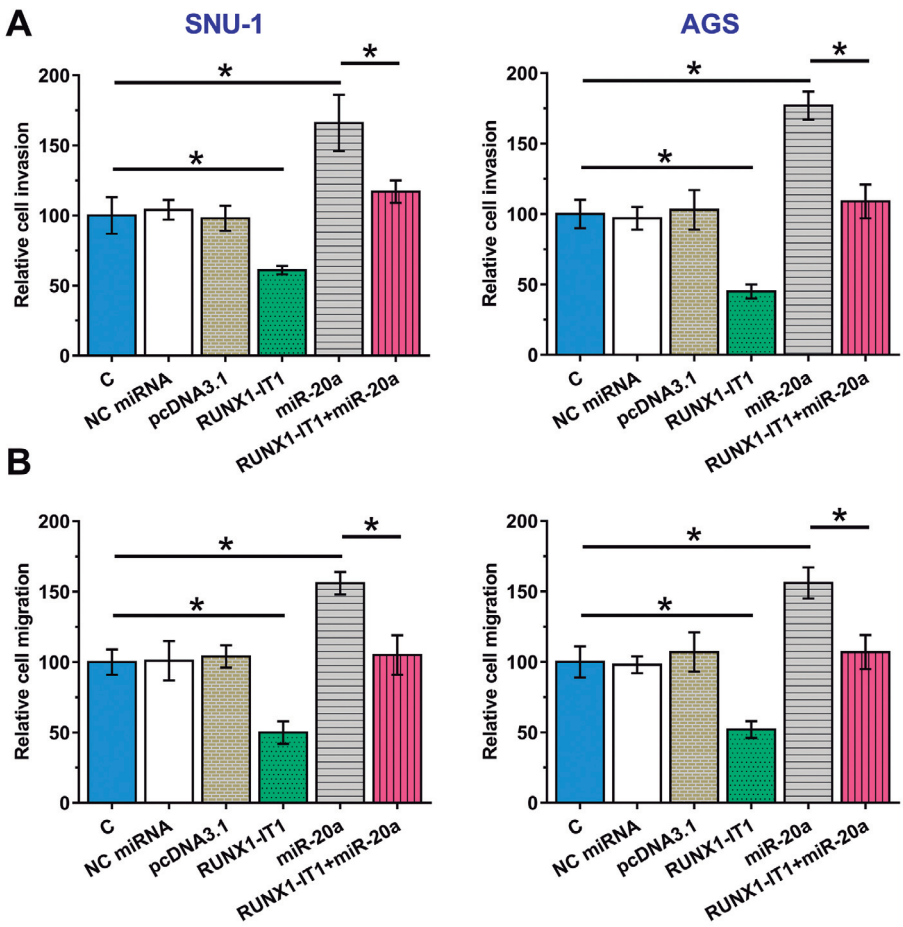

Fig. 4. RUNX1-IT1 overexpression inhibited the invasion and migration of SNU-1 and AGS cells through miR-20a. Transwell assays were performed to analyze the effects of RUNX1-IT1 and miR-20a overexpression on the invasion **(A)** and migration **(B)** of SNU-1 and AGS cells. Data of multiple transfection groups were expressed as mean±SD values of three independent replicates. *, $p<0.05$.

20a were downregulated in GC. Moreover, overexpression of miR-20a decreased cancer cell invasion and migration.

Interestingly, overexpression of RUNX1-IT1 only reduced the production of mature miR-20a. Therefore, RUNX1-IT1 may prevent miR-20a from maturation to play its tumor suppressive role in GC. Although we did not perform *in vivo* experiments, we observed the significant correlation between RUNX1-IT1 and mature miR-20a across GC tissues. Therefore, RUNX1-IT1 may also repress the maturation of miR-20a in the human body. Future studies are needed to explore the role of RUNX1-IT1 in miRNA biogenesis (Romero-Cordoba et al., 2014).

Conclusion

In conclusion, RUNX1-IT1 is upregulated in GC, and it may suppress the maturation of miR-20a to suppress cell invasion and migration.

Conflict of Interest Statement. No competing interests to declare.

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