

Long non-coding RNA-ZNF281 promotes cancer cell migration and invasion in gastric cancer via downregulation of microRNA-124

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Abstract. It has previously been determined that long non-coding (lnc)RNA-zinc finger protein (ZNF)281 serves an oncogenic role in breast cancer; however, the role of lncRNA-ZNF281 in other cancer types is yet to be elucidated. The present study aimed to analyze the role of ZNF281 in gastric cancer by characterizing its activity in cancerous tissues and normal tissues using RT-qPCR. Overexpression experiments were also performed to investigate the interaction between ZNF281 and miR-124, and Transwell assays were conducted to analyze cell invasion and migration. The present study revealed that lncRNA-ZNF281 was upregulated, and that microRNA (miR)-124 was downregulated, in cancerous tissues compared with that in the paired adjacent healthy tissues of patients with gastric cancer. In addition, the expression levels of lncRNA-ZNF281 and miR-124 exhibited a significant inverse association. Furthermore, *in vitro* cell experiments determined that lncRNA-ZNF281 overexpression resulted in miR-124 inhibition, yet miR-124 overexpression did not influence lncRNA-ZNF281 expression. lncRNA-ZNF281 expression level was also associated with the clinical stage of the patient. Bioinformatics analysis revealed that lncRNA-ZNF281 may target the base pairs in the hairpin loop of the miR-124 precursor. Subsequent *in vitro* cell experiments indicated that lncRNA-ZNF281 overexpression resulted in promoting the migration and invasiveness of gastric cancer cells, while miR-124 over-expression led to its inhibition. In addition, miR-124 overexpression partially recovered the effects of lncRNA-ZNF281 overexpression. Therefore,

lncRNA-ZNF281 may promote cancer cell migration and invasion in gastric cancer via downregulation of miR-124.

Introduction

Despite progress in both the treatment and prevention of numerous cancer types, gastric cancer is still a prevalent malignancy in both males and females (1). In 2018, there were 1,033,701 new diagnoses of gastric cancer alone (5.7% of all cancer diagnoses), which resulted in 782,685 mortalities (8.2% of all cancer-associated mortalities) (2). Lymph node metastasis and distant metastasis are common in patients with gastric cancer and effective therapeutic approaches targeting metastatic gastric cancer are ineffective (3,4), resulting in a poor prognosis (3,4). *Helicobacter pylori* infections and certain dietary structures (including alcohol intake and vitamin C deficiency) are major risk factors for gastric cancer (5,6). However, the pathogenesis of this is poorly characterized.

Genetic alterations significantly contribute to the development and pathogenesis of gastric cancer (7). Non-coding RNAs (ncRNAs), such as long ncRNAs (lncRNAs) (>200 nt) and microRNAs (miR/miRNAs) regulate gene expression to participate in the development of diverse types of cancer, including gastric cancer (8-10). A recent study showed that lncRNAs can interact with miRNAs to regulate cancer cell behaviors (11). lncRNA-zinc finger protein (ZNF)281 is a recently identified cancer-associated lncRNA in glioma (12). Our preliminary bioinformatics analysis indicated that lncRNA-ZNF281 can bind the loop region of miR-124 precursor, while miR-124 suppresses gastric cancer (13). The present study was performed to explore the interaction between lncRNA-ZNF281 and miR-124 in gastric cancer and to examine the differential expression of ZNF281 in gastric cancer using reverse transcription-quantitative (RT-q)PCR. Moreover, the interaction between ZNF281 and miR-124 was analyzed via overexpression experiments. Transwell assays were conducted to analyze the roles of ZNF281 and miR-124 in regulating cell invasion and migration. It was revealed that lncRNA-ZNF281 may promote cancer cell migration and invasion in gastric cancer cells via the downregulation of miR-124.

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Materials and methods

Gastric patients. A total of 72 patients with gastric cancer (44 males and 28 females; median age, 50.1±6.3 years; range, 33–67 years) were selected from 188 cases of gastric cancer admitted to the Second People's Hospital of Liaocheng (Linqing, China) between March 2015 and April 2019. Of the 72 patients, there were 38 cases of adenocarcinoma and 34 cases of carcinoma. The current study was approved by the Ethics Committee of the aforementioned hospital. The inclusion criteria were as follows: i) Patients with newly diagnosed gastric cancer; and ii) a diagnosis made using a histopathological test (the gold standard). The exclusion criteria were as follows: i) Patients with recurrent gastric cancer; ii) prior initiation of therapy; and iii) other diagnosed clinical disorders. According to the clinical data, the patients were classified according to the American Joint Committee on Cancer staging system (14). The results revealed that there were a total of 12, 18, 22 and 20 patients at clinical stages I, II, III and IV, respectively. The principle of the experimental design was explained to all 72 patients, and all provided written informed consent.

Gastric tissue specimens and cell lines. Prior to therapy initiation, a gastric biopsy was performed under the guidance of MRI to collect both tumor and non-tumor gastric tissues from all included patients. Non-tumor samples were collected ≤3 cm away from the cancerous tissues, and all samples were validated via histopathological examination. Additionally, two cell lines, AGS (gastric adenocarcinoma) and SNU-1 (gastric carcinoma) from the American Type Culture Collection (ATCC), were used in the present study. These cells were cultured in a mixture of 10% FBS (ATCC) and 90% F-12K Medium (ATCC), at 95% humidity, 37°C and 5% CO₂.

Transient cell transfections. The pCDNA3.1 vector was used to construct an lncRNA-ZNF281 expression vector (Sangon Biotech Co., Ltd.). Negative control (NC; non-targeting control) miRNA (5'-GUAGUCGAUGCACGAUCGUAU-3') and miR-124 mimic (5'-CGUGUUCACAGCGGACCUUGAU-3') were also purchased from Sangon Biotech Co., Ltd. AGS and SNU-1 cells were harvested at 80% confluence, followed by transfection with 10 nM vector (empty vector as NC group) and 50 nM miRNA (NC miRNA as NC group) into 1×10⁶ cells. All transfections were mediated by Lipofectamine[®] 2000 reagent (Sangon Biotech Co., Ltd.). The control (C) in all transfections was untransfected cells. Cells were harvested at 24 h post-transfection to perform the following experiments.

RNA interaction prediction. IntaRNA 2.0 (<http://rna.informatik.uni-freiburg.de/IntaRNA/Input.jsp>) was used to predict the interaction between lncRNA-ZNF281 and miR-124 precursor. For the analysis, the lncRNA-ZNF281 sequence was used as the long sequence, and the miR-124 precursor sequence was used as the short sequence.

RT-qPCR. All tissue samples were stored in liquid nitrogen. AGS and SNU-1 cells were harvested at 24 h post-transfection and cells were counted. Total RNAs in 0.03-g tissue samples and 1×10⁵ cells were extracted using RNeasy reagent

(Sigma-Aldrich; Merck KGaA). To harvest miRNAs, 85% ethanol was used to precipitate and wash RNA samples. Total RNA was digested using DNase I for 90 min at 37°C, before being reverse transcribed into cDNA using TruScript Reverse Transcriptase kit (Norgen Biotek Corp.). To measure the expression levels of lncRNA-ZNF281, all qPCR assays were performed using Luna[®] Universal One-Step RT-qPCR kit (SYBR; New England BioLabs, Inc.) with 18S rRNA as an endogenous control. Primer sequences were as follows: lncRNA-ZNF281 forward, 5'-GAGGACACATAGTGGAGAAAG-3' and reverse, 5'-TGAGACAACACAGCCAGATTAC-3'; 18S rRNA forward, 5'-CTACCACATCCAAGGAAGC-3' and reverse, 5'-TTTTCGTCCTACCTCCCC-3'. To measure the expression levels of mature miR-124, the addition of poly(A), miRNA reverse transcription and qPCR assays were also performed using the All-in-One[™] miRNA RT-qPCR Reagent kit (GeneCopoeia, Inc.). The miR-124 forward sequence was: 5'-CGUGUUCACAGCGGACCUU-3'. Reverse primer and U6 primers were included in the All-in-One[™] miRNA RT-qPCR Reagent kit (cat. no. QP015; GeneCopoeia, Inc.) with U6 used as an endogenous control. All Cq values were processed using 2^{-ΔΔCq} method (15) and each PCR was repeated in triplicate. All PCR reactions conditions were: 95°C for 1 min, followed by 40 cycles of 95°C for 10 sec and 57°C for 40 sec.

Transwell assays. AGS and SNU-1 cells were harvested at 24 h post-transfection. Transwell assays were performed to analyze the effects of transfection with various molecules on the invasion and migration of these cell lines. Briefly, single-cell suspensions were prepared by mixing 3×10⁴ cells with 1 ml serum-free F-12K Medium. Membranes were coated with Matrigel at 37°C for 6 h before being used in the invasion assay, and uncoated membranes were used for the migration assay. The upper Transwell chamber was loaded with 0.1 ml single-cell suspension, while the lower chamber was filled with a mixture of 80% F-12K Medium and 20% FBS. Cells were incubated for 12 h under the aforementioned conditions. After that, membranes were stained using 0.1% crystal violet (Sigma-Aldrich; Merck KGaA) at room temperature for 20 min, and cells were counted under a light microscope (magnification, 40x).

Statistical analysis. All experiments were performed in 3 independent biological replicates. The mean was then calculated and was used in all subsequent statistical analyses. GraphPad Prism 6 (Graphpad Software, Inc.) software was used for all data analysis. Associations were analyzed using Linear regression. Differences between 2 groups were analyzed using a Student's t-test and differences between ≥3 groups were compared using ANOVA (one-way) followed by Tukey's post hoc test. The 72 gastric cancer patients were divided into high- and low-lncRNA-ZNF281 or -miR-124 expression level groups (n=36), with the median expression level of the respective molecules in tumor tissues used as cutoff values (4.72 and 2.12 for lncRNA-ZNF281 and miR-124, respectively). The χ^2 test was performed to analyze the association between the expression levels of lncRNA-ZNF281 and miR-124 with patient clinical data. P<0.05 was considered to indicate a statistically significant difference.

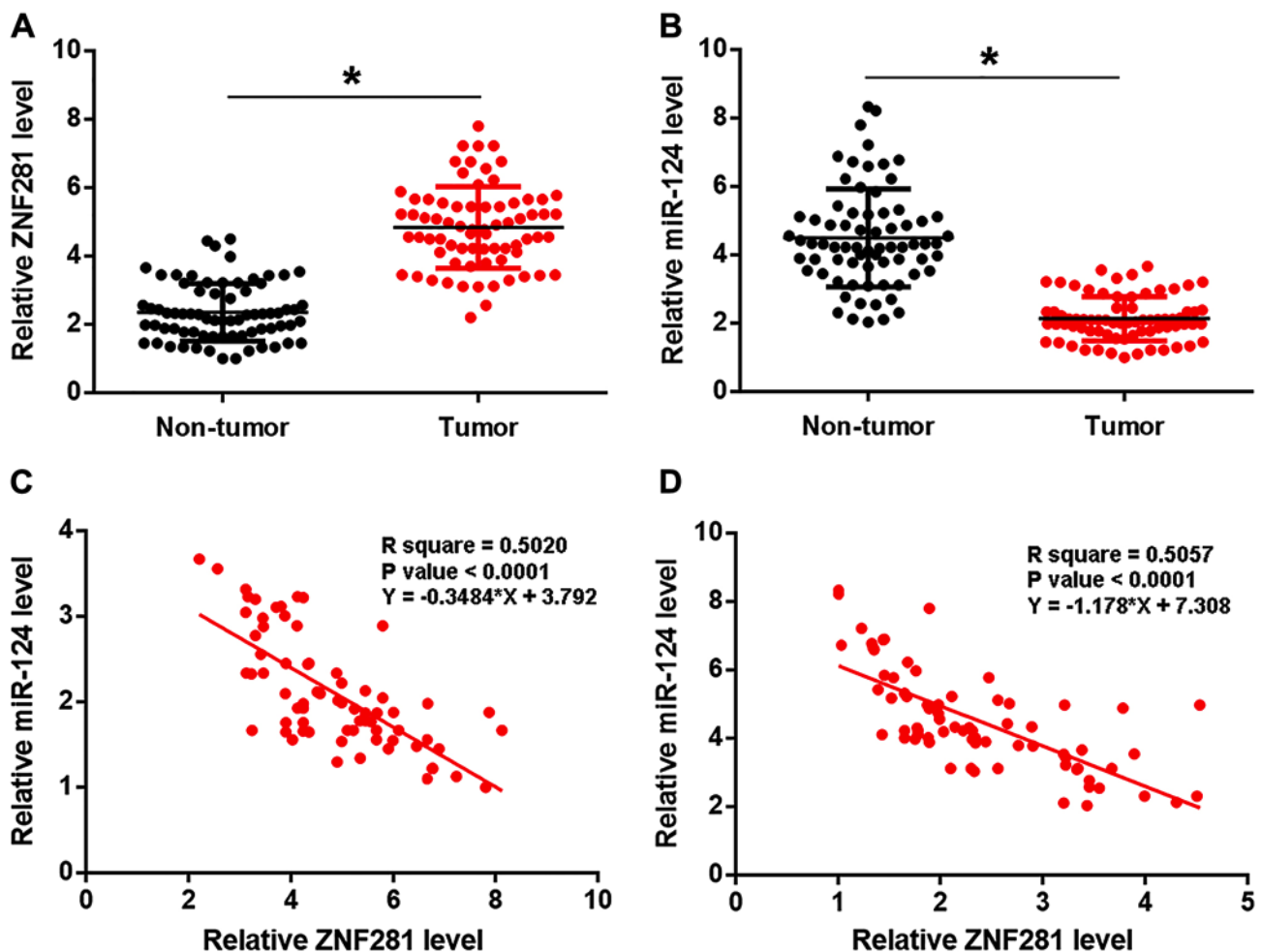


Figure 1. lncRNA-ZNF281 and miR-124 are inversely correlated in tumor tissues. Expression levels of (A) lncRNA-ZNF281 and (B) miR-124 in two types of tissues were measured using qPCR and compared using the paired Student's t-test. Associations between lncRNA-ZNF281 and miR-124 in (C) tumor and (D) non-tumor tissues were analyzed by linear regression. Data are presented as the mean values of triplicate experiments. * $P < 0.05$. lncRNA, long non-coding RNA; ZNF281, zinc finger protein 281; miR, microRNA; qPCR, quantitative PCR.

Results

lncRNA-ZNF281 expression is inversely correlated with miRNA expression in gastric tumor tissues. Expression levels of lncRNA-ZNF281 and miR-124 in two types of tissues were measured using qPCR and compared using the paired Student's t-test. lncRNA-ZNF281 expression was upregulated (Fig. 1A), while miR-124 was downregulated (Fig. 1B) in tumor tissues, compared with adjacent non-tumor tissues ($P < 0.05$). Subsequently, the association between lncRNA-ZNF281 and miR-124 expression was analyzed using linear regression and revealed a significant and inverse association in both tumor (Fig. 1C) and adjacent non-cancerous (Fig. 1D) tissues. The χ^2 test revealed that lncRNA-ZNF281 and miR-124 expression levels were not significantly associated with patient age, sex or gastric cancer subtype (adenocarcinoma or carcinoma), but were significantly correlated with clinical stage ($P < 0.05$; Tables I and II).

lncRNA-ZNF281 and miR-124 expression levels are significantly associated with clinical stage. Based on the American Joint Committee on Cancer staging system, the 72 patients presented with a total of 12, 18, 22 and 20 cases at

clinical stages I, II, III and IV, respectively. Expression levels of lncRNA-ZNF281 and miR-124 were compared between the 4 clinical stages by performing a one-way ANOVA, followed by Tukey's post hoc test. It was revealed that the expression levels of the lncRNA were higher in patients at a more advanced clinical stage ($P < 0.05$; Fig. 2A). Conversely, expression levels of miR-124 decreased significantly in patients at a more advanced clinical stage ($P < 0.05$; Fig. 2B).

lncRNA-ZNF281 may act as a molecular sponge of the miR-124 precursor molecule, downregulating miR-124 expression. To further investigate the interactions between lncRNA-ZNF281 and miR-124, AGS and SNU-1 cells were transfected with an lncRNA-ZNF281 expression vector or miR-124 mimic. Expression levels of lncRNA-ZNF281 and miR-124 were measured at 24 h post-transfection. Compared with the NC (empty vector or NC miRNA transfection) and C groups, expression levels of lncRNA-ZNF281 and miR-124 were significantly increased in transfected cells ($P < 0.05$; Fig. 3A). Moreover, lncRNA-ZNF281 overexpression mediated the downregulation of miR-124, compared with both control groups ($P < 0.05$; Fig. 3B), while miR-124 overexpression did not significantly influence lncRNA-ZNF281 expression

Table I. Association between the expression level of lncRNA-ZNF281 and patient clinicopathological data.

Characteristic	Cases, n	High expression, n	Low expression, n	χ^2 value	P-value
Age, years				0.48	0.50
>50	37	20	17		
<50	35	16	19		
Sex				0.94	0.33
Male	44	24	20		
Female	28	12	16		
AJCC stage				14.67	<0.01
I	12	3	9		
II	18	4	14		
III	22	15	7		
IV	20	14	6		
Subtypes				0.89	0.35
Adenocarcinoma	38	17	21		
Carcinoma	34	19	15		

lncRNA, long non-coding RNA; ZNF281, zinc finger protein 281.

Table II. Association between the expression level of miR-124 with patient clinicopathological data.

Characteristic	Cases, n	High expression, n	Low expression, n	χ^2 value	P-value
Age, years				0.06	0.81
>50	37	18	19		
<50	35	18	17		
Sex				0.23	0.63
Male	44	21	23		
Female	28	15	13		
AJCC stages				11.73	<0.01
I	12	8	4		
II	18	14	4		
III	22	8	14		
IV	20	6	14		
Subtypes				0.22	0.64
Adenocarcinoma	38	20	18		
Carcinoma	34	16	18		

miR, microRNA; AJCC, American Joint Committee on Cancer.

(Fig. 3C). Bioinformatics analysis (performed using IntaRNA) revealed that lncRNA-ZNF281 may bind to a complementary base sequence in the hairpin loop of miR-124 precursor (Fig. 3D).

lncRNA-ZNF281 suppresses the invasion and migration of gastric cancer cells, via miR-124 downregulation. Transwell invasion and migration assays were performed to analyze the effects of lncRNA-ZNF281 and miR-124 overexpression on the invasion (Fig. 4A) and migration (Fig. 4B) of both AGS and SNU-1 cells. Compared with the NC and C groups, lncRNA-ZNF281 overexpression led to promotion of both

migration and invasion of gastric cancer cells, while miR-124 overexpression led to their inhibition ($P<0.05$). In addition, miR-124 overexpression partially recovered the action of lncRNA-ZNF281 overexpression ($P<0.05$).

Discussion

The present study investigated the functional role of lncRNA-ZNF281 in the progression of gastric cancer, and determined that lncRNA-ZNF281 was downregulated in gastric cancer tissues, promoting the invasion and migration of cells via downregulation of miR-124 expression.

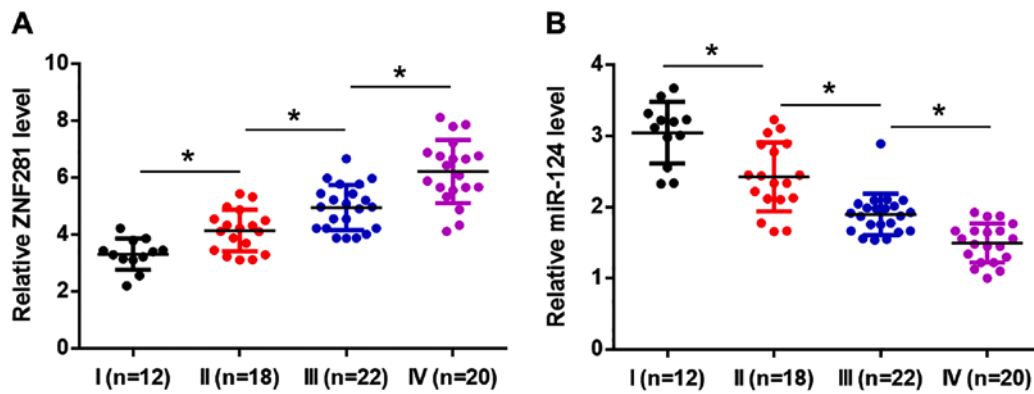


Figure 2. lncRNA-ZNF281 and miR-124 expression levels were significantly affected by clinical stages. Based on the AJCC stage, the 72 patients included 12, 18, 22 and 20 cases at clinical stage I, II, III and IV. Expression levels of (A) lncRNA-ZNF281 and (B) miR-124 were compared between the 4 clinical stages by performing ANOVA (one-way) in combination with Tukey's post hoc test. *P<0.05. lncRNA, long non-coding RNA; ZNF281, zinc finger protein 281; miR, microRNA; AJCC, American Joint Committee on Cancer.

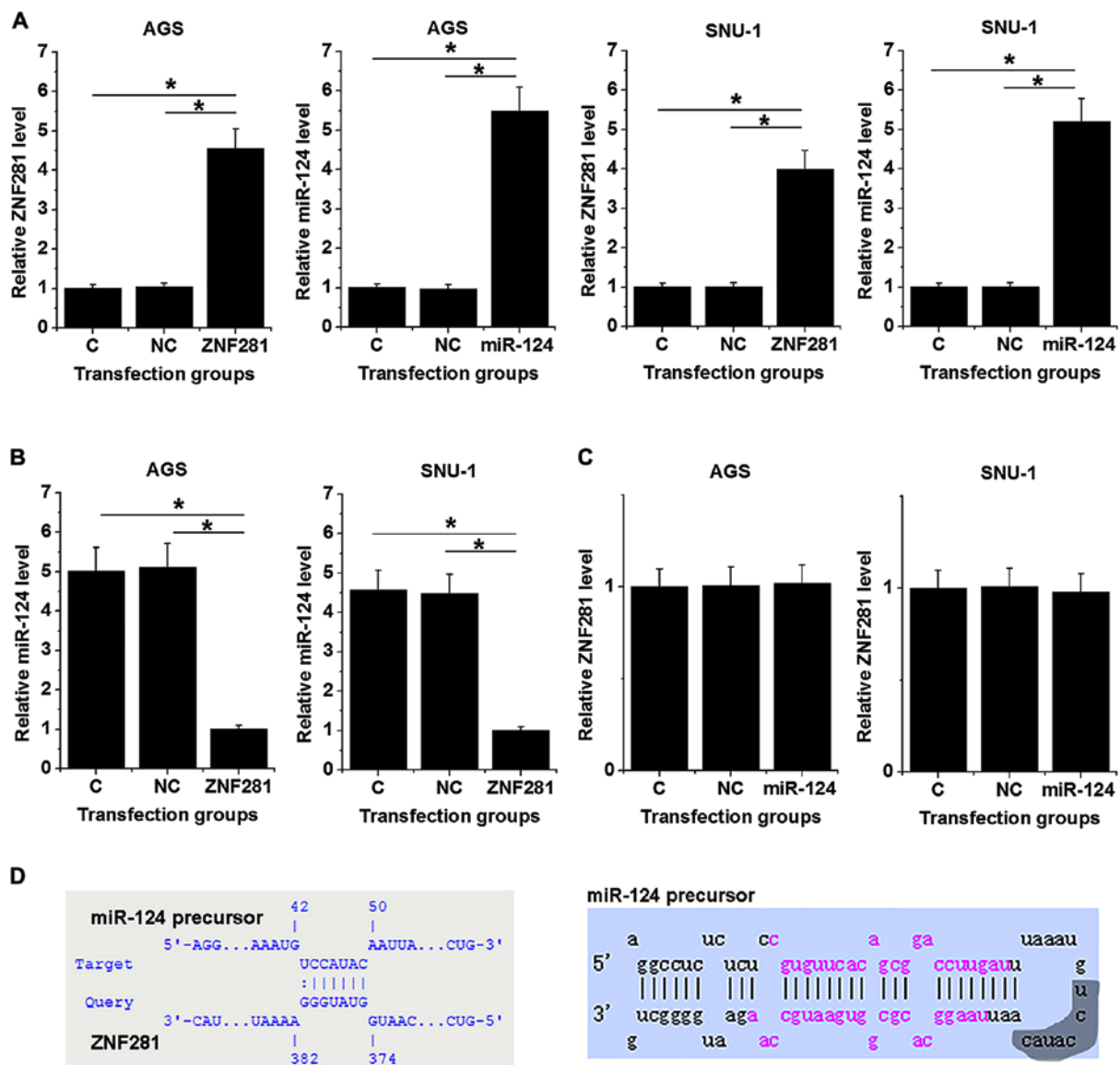


Figure 3. lncRNA-ZNF281 may serve as a sponge miR-124 precursor to downregulate mature miR-124. To further investigate the interactions between lncRNA-ZNF281 and miR-124, AGS and SNU-1 cells were transfected with lncRNA-ZNF281 expression vector or miR-124 mimic. (A) Overexpression of lncRNA-ZNF281 and miR-124 was confirmed using qPCR at 24 h post-transfection. (B) Effect of lncRNA-ZNF281 overexpression on miR-124 expression and (C) effect of miR-124 overexpression on lncRNA-ZNF281 expression was analyzed using qPCR. (D) Interaction between lncRNA-ZNF281 and miR-124 precursor was predicted using IntaRNA and the bases of miR-124 precursor involved in base pairing are shown highlighted in gray. Mean values of triplicate experiments are presented. *P<0.05. lncRNA, long non-coding RNA; ZNF281, zinc finger protein 281; miR, microRNA; qPCR, quantitative PCR.

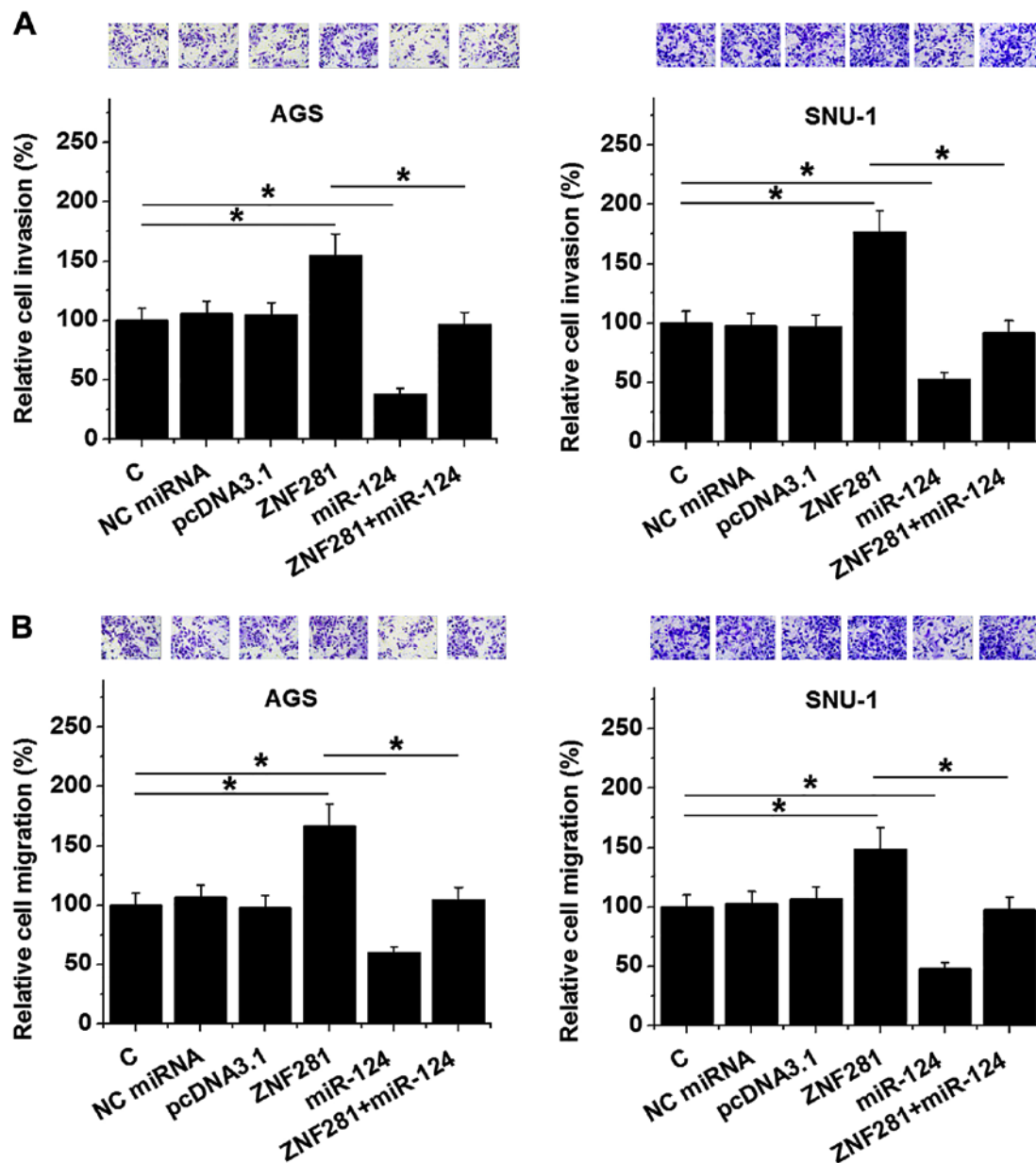


Figure 4. lncRNA-ZNF281 suppressed the invasion and migration of gastric cancer cells through miR-124. Transwell invasion and migration assays were performed to analyze the effects of lncRNA-ZNF281 and miR-124 overexpression on the (A) invasion and (B) migration of both AGS and SNU-1 cells. Mean values of triplicates are presented. * $P < 0.05$. Cells were stained using 0.1% crystal violet at room temperature for 20 min, and images were obtained under $\times 40$ magnification. lncRNA, long non-coding RNA; ZNF281, zinc finger protein 281; miR, microRNA.

The majority of lncRNAs serve a similar role in numerous cancer types; for instance, lncRNA HOX transcript antisense RNA is upregulated in all cancer types, and regulates chromatin dynamics to promote cancer progression (16). However, it has also been observed that lncRNAs sometimes exhibit opposite functions in certain cancer types. For example, lncRNA taurine upregulated gene 1 (TUG1) is downregulated in glioma and acts as a tumor suppressor by promoting glioma cell apoptosis (17). TUG1 is also upregulated in osteosarcoma and promotes cancer cell proliferation (18). Furthermore, lncRNA-ZNF281 was downregulated in glioma and inhibited cancer cell invasion (12). By contrast, the present study revealed that lncRNA-ZNF281 was upregulated in gastric cancer tissues and promoted the invasion and migration of cancer cells.

Therefore, lncRNA-ZNF281 may serve different, or even opposite roles depending on cancer type.

It has been revealed that lncRNAs are able to act as molecular sponges of miRNAs, attenuating their effects on downstream genes (19); however, the sponging of mature miRNAs by lncRNAs does not affect the expression levels of miRNAs. Notably, in the present study, it was discovered that lncRNA-ZNF281 downregulated miR-124 expression. It has previously been reported that miR-124 can repress Snail2 to inhibit cancer cell invasion in gastric cancer (13). Therefore, miR-124 may represent a link between Snail2 and lncRNA-ZNF281. There was no indication of significant interaction between mature miR-124 and lncRNA-ZNF281; instead, lncRNA-ZNF281 was revealed to form a strong base pairing with the loop region of the secondary structure of

miR-124 precursor. It is known that the hairpin structure of miRNA precursors is critical for miRNA maturation (20). Therefore, lncRNA-ZNF281 may prevent the formation of the hairpin structure of miR-124 precursor and suppress its maturation, thereby downregulating the expression level of mature miR-124. However, more experiments are needed to further validate this conclusion.

Notably, as a result of a lack of available resources, the current study failed to produce an miR-124 mutant, which may help to verify the interaction between lncRNA-ZNF281 and miR-124. This should be incorporated into future studies. In addition, preliminary cell proliferation assay results demonstrated no effects of lncRNA-ZNF281 overexpression on the proliferation of gastric cancer cells (data not shown). Therefore, lncRNA-ZNF281 may only regulate certain behaviors of gastric cancer cells, and may not influence factors associated with proliferation.

In conclusion, the present study revealed that lncRNA-ZNF281 is upregulated in gastric cancer and able to downregulate miR-124 expression, resulting in the suppression of cancer cell invasion and migration.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

SL, HG and PL designed and performed experiments. YH, JW, XL and JZ collected and analyzed data. SL drafted the manuscript, which was approved by all authors.

Ethics approval and consent to participate

The current study was approved by the Ethics Committee of the Second People's Hospital of Liaocheng (Linqing, China) and written informed consent was obtained from each patient.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interest.

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