MicroRNA-26b acts as an antioncogene and prognostic factor in cervical cancer

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Abstract. Cervical cancer is the second most frequent malignant neoplasm in women all over the world. MicroRNA-26b (miR-26b) has been reported to be downregulated and play a great role in many malignancies, nevertheless, there are scarce studies on cervical cancer. The purpose of the present study was to detect how miR-26b is involved in cervical carcinoma. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was utilized to detect the expression levels of miR-26b and Jagged1 (JAG1) mRNA. Transwell assay was applied to calculate the cell migration and invasion capacity. Luciferase reporter assay was employed to determine JAG1 as a target of miR-26b. The results revealed that miR-26b is downregulated in cervical cancer tissues and cells compared with paracancerous tissues and normal cervical epithelial cells. The low expression of miR-26b in cervical cancer demonstrated that miR-26b inhibits cell migration and invasion, as measured by Transwell assay. JAG1 was verified to be a target of miR-26b and have a negative correlation with miR-26b, as detected by luciferase reporter assay. In addition, miR-26b was found to suppress cell migration and invasion via mediating JAG1 expression, which impact is partially reversed by JAG1. In conclusion, miR-26b suppresses cell migration and invasion of cervical cancer through directly targeting JAG1. It is suggested that miR-26b/JAG1 axis may present a new target for the treatment of cervical cancer.

Introduction

Cervical cancer, with cervical squamous cell carcinoma being the most common type, is the second most frequent malignant neoplasm in women worldwide. There are ~500,000 patients diagnosed with cervical cancer and 200,000 deaths estimated each year (1). The exact reasons for cervical cancer occurrence, such as infected high-risk human papillomavirus (HPV) and oncogenes or tumor suppressor gene mutations, are not fully understood (2). Thus, the identification of novel molecular biomarkers would be helpful in predicting the occurrence and prognosis of cervical cancer.

MicroRNAs (miRNAs) are 22-28 oligonucleotide-long RNAs that mediate gene expression via targeting the 3'-untranslated regions (3'UTR) of gene mRNA at post-transcriptional level. Therefore miRNAs could induce mRNA degradation or inhibit gene expression (3-12). Recent studies have expounded that miRNAs may take part in ~60% of all human gene post-transcriptional regulations and control the oncogenic or tumor-suppressive activities of their target genes. miR-26b, a member of miR-26 family, has been reported to be a tumor suppressor in oral squamous cell carcinoma, colorectal cancer, breast cancer and glioma (13-17). miR-26b could suppress tumor cell growth through targeting PTGS2 in breast cancer (15). Wu *et al* have demonstrated that in glioma cells miR-26b inhibits cell migration and invasion (17). In addition, miR-26b could suppress lens fibrosis and cataract through mediating Jagged1 (JAG1), which belongs to Jagged/Notch signaling pathway.

JAG1, a Notch ligand of Notch signaling pathway, binds to Notch receptor which causes a conformational transformation and allows a secondary cutting by tumor necrosis factor-α converting enzyme (18). Furthermore, many miRNAs interact with JAG1 and affect tumor progression. miR-26b suppresses lens fibrosis and cataract via targeting JAG1 (19).

Although the anti-proliferation functions of miR-26b have been reported in cervical cancer, its role on cell migration and invasion still needs exploring. In the present study, we demonstrate that miR-26b mediates JAG1 expression, reducing the cervical cancer cell migration and invasion ability through inhibiting JAG1 expression. Moreover, the decrease of migration and invasion ability by miR-26b could be weakened by transfected JAG1. In addition, the 5-year overall and disease free-survival rates are found to be lower when miR-26b expression is low, which predicts poor prognosis. Thus, miR-26b mediates cervical cell migration and invasion by inhibiting JAG1 expression.

Patients and methods

Patients and tumor samples. Paired cervical cancer and paracancerous tissues were obtained from 54 patients with cervical

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cancer who were hospitalized in Shangluo Central Hospital (Shangluo, China) from 2015 to 2017. Before analysis, all specimens were frozen in liquid nitrogen immediately after surgery and stored at -80˚C. For this cohort, 30 patients were diagnosed at early stage (0/I/II), while the others were diagnosed at advance stage (III/V), according to the International Federation of Gynecology and Obstetrics (FIGO). Stage grouping and the detailed clinical information are shown in Table I. None of the patients had undergone chemotherapy or radiotherapy before surgery. For all specimens informed consent was obtained from the patients and the study was approved by the Ethics Committee of Shangluo Central Hospital.

Cell lines and culture conditions. Human cervical cancer cell lines HeLa (cat. no. CCL-2), JAR (cat. no. HTB-144), and normal cervical immortalized squamous cells Ect1/E6E7 (cat. no. CRL-2614) were purchased from American Type Culture Collection (ATCC; Rockville, MD, USA). The cells were cultured in a cell incubator at 37˚C using RPMI-1640 medium (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Carlsbad, CA, USA). Both media were supplemented with penicillin-streptomycin (final concentration of penicillin was 100 U/ml and of streptomycin was 0.1 mg/ml; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China).

RNA isolation and RT-qPCR. Total RNA and total miRNA were extracted utilizing TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and miRcute miRNA isolation kit (Tiangen Biotech Co., Ltd., Beijing, China), respectively. After measuring concentration, PrimeScript™ II 1st Strand cDNA Synthesis kit (Takara Biotechnology Co., Ltd., Dalian, China) was used to synthesize cDNA. In addition, SYBR Premix kit and SYBR PrimeScript miRNA RT-PCR kit (both from Takara Bio, Inc., Otsu, Japan) were employed to perform qPCR. The primer sequences used were: miR-26b forward, 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGAGCCA-3' and reverse, 5'-CGCCCTGTTCTCCATTACTT-3'; JAG1 forward, 5'-ATCGTGCTGCCTTTCAG TTT-3' and reverse, 5'-GATCATGCCCGAGTGAGAA-3'; GAPDH forward, 5'-CCACTCCTCCACCTTTGAC-3' and reverse, 5'-ACCCTGTTGCTGTAGCCA-3'; and U6 forward, 5'-CTTGGCAGCACATATACT-3' and reverse, 5'-AAAATATGGAACGCTTCACG-3'. The thermocycling conditions were as follows: 2 min at 95˚C, followed by 40 cycles of 30 sec at 95˚C and 45 sec at 60˚C. The expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method (20). GAPDH and U6 were utilized to normalize mRNA and miRNA, respectively.

Protein extraction and western blotting. Specific cells were lysed by RIPA lysis buffer with 1% protease inhibitor phenylmethanesulfonyl fluoride (PMSF; Beyotime Institute of Biotechnology, Shanghai, China) to obtain total proteins. After quantified with BCA Protein Assay kit (Beijing Solarbio Science & Technology Co., Ltd.), 50 μg of proteins were added into each polyacrylamide gel electrophoresis well and electrophoresis was carried out to separate all proteins. Then, the blots were transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and blocked with 5% skim milk powder at room temperature for 1 h. The membrane was incubated with rabbit JAG1 polyclonal antibody (cat. no. PAB807Mu01; 1:500; Wuhan USCN Business Co., Ltd., Wuhan, China) and mouse GAPDH momoclonal antibody (cat. no. sc-32233; 1:2,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 4˚C overnight. Then, the membrane was incubated with rabbit antibody labeled with HRP (cat. no. 5571; 1:5,000; Cell Signaling Technology, Inc., Danvers, MA, USA) as secondary antibody for 2 h at room temperature. ECL Western Blotting Detection System (BestBio, Beijing, China) was employed to analyze the protein bands.

Transwell assay. The capacity of cell migration and invasion were measured with Transwell assay. The Transwell chambers with or without Matrigel (Clontech Laboratories, Inc., Mountainview, CA, USA) were put into a 24-well plate, thus forming upper and lower chambers. Cell suspension (200 μl) with density of $1x10^5$/ml was plated in the upper chamber and the medium of the suspended cells was free of FBS. The lower chamber was added with 400 μl RPMI-1640 supplemented with 20% FBS. After incubation for 24 h at 37˚C, the cells which moved to the lower surface were stained with 0.5%

Table I. Clinicopathological features and miR-26b expression in 54 paired cervical cancer tissues.

Clinicopathological features	Cases (n=54)	miR-26b expression		P-value[a]
		High (%)	Low (%)	
Age (years)				0.860
≤50	38	18 (47.4)	20 (52.6)	
>50	16	8 (50.0)	8 (50.0)	
Tumor size (mm)				0.151
≤4.0	32	18 (56.3)	14 (43.7)	
>4.0	22	8 (36.4)	14 (63.6)	
FIGO stage				0.033[a]
0-II	30	20 (66.7)	10 (33.3)	
III-IV	24	10 (41.7)	14 (58.3)	
Lymph node metastasis				0.025[a]
No	31	19 (61.3)	12 (38.7)	
Yes	23	7 (30.4)	16 (69.6)	
Histology				0.336
Squamous	48	22 (45.8)	26 (54.2)	
Adenocarcinoma	6	4 (66.7)	2 (33.3)	
SCC-Ag (ng/l)				0.0327[a]
≤4	44	24 (54.5)	20 (45.5)	
>4	10	2 (20.0)	8 (80.0)	
JAG1				0.030[a]
Negative	21	14 (66.6)	7 (33.3)	
Positive	33	12 (36.3)	21 (63.6)	

[a]P<0.05, statistically significant difference. miR-26b, microRNA-26b; FIGO, International Federation of Gynecology and Obstetrics; SCC-Ag, squamous cell carcinoma antigen; JAG1, Jagged1.

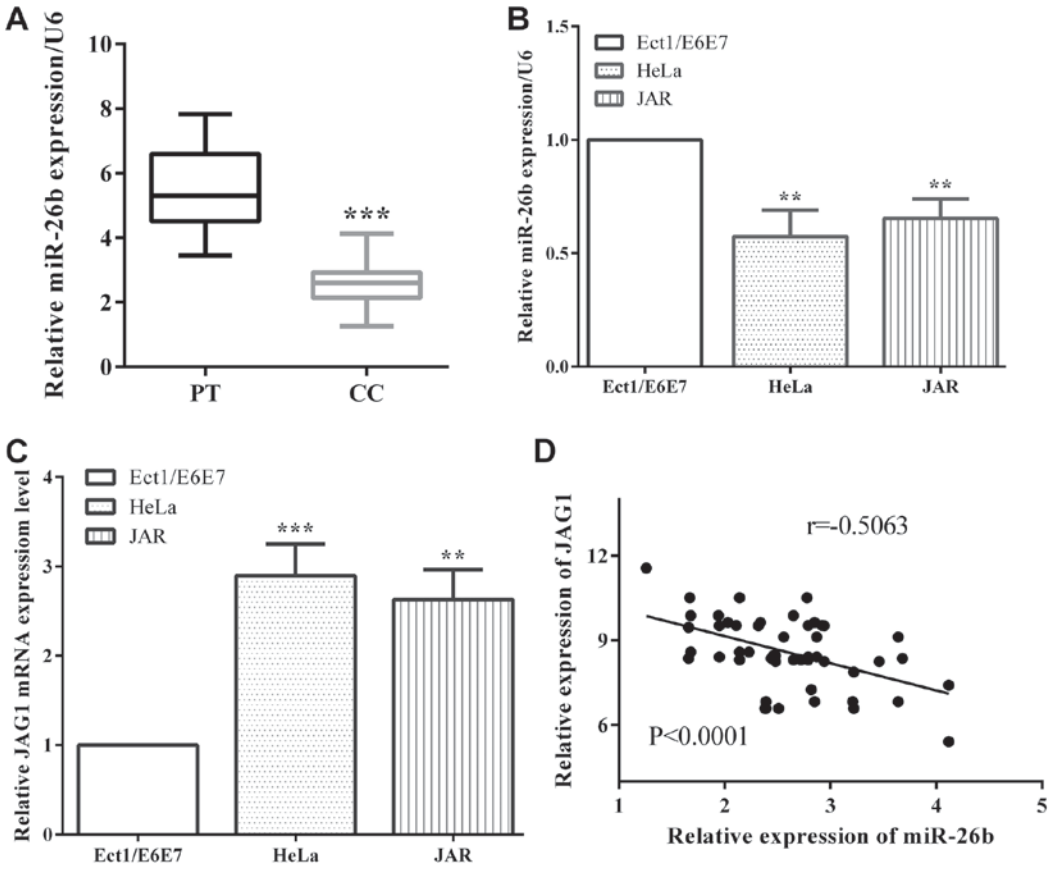

Figure 1. RT-qPCR detection of miR-26b and JAG1 expression levels in cervical cancer tissues and cells. (A) The relative expression level of miR-26b was determined in CC tissues and PT by RT-qPCR (normalized by U6). (B) miR-26b expression levels in HeLa and JAR are expressed as relative to that of Ect1/E6E7 cells. (C) JAG1 mRNA expression levels in HeLa and JAR are compared to that of normal cervical epithelial cells Ect1/E6E7. (D) Correlation between miR-26b and JAG1 mRNA expression levels is presented. **P<0.01, ***P<0.001. miR-26b, microRNA-26b; JAG1, Jagged1; CC, cervical cancer; PT, paracancerous tissues.

crystal violet for 30 min and the cells on the upper chamber were removed. The cells in the lower chamber were observed with a microscope (BX51 Olympus; Olympus Corp., Tokyo, Japan) and counted at five random fields.

Plasmid construction and luciferase reporter assay. JAG1 was predicted to be a target gene of miR-26b by TargetScan online software (http://www.targetscan.org/vert_71/) and the two binding sites were located at 989-995 and 1,248-1,255 on JAG1 3'UTR. Genomic DNA acted as template to amplify JAG1 3'UTR sequences and then the 3'UTR sequences were cloned to pmirGlo plasmid (named pmirGlo-JAG1-WT). The binding sites were mutated and inserted in pmirGlo vector, named as pmirGlo-JAG1-MUT site1 and site2 (MUT S1 and MUT S2). In addition, the cells were co-transfected with the recombinant reporter plasmids (pmirGlo-JAG1-WT and pmirGlo-JAG1-WUT) and miR-211 mimic or its scramble negative controls using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). After transfection for 48 h, luciferase activities were detected using Dual-Glo Luciferase Assay System (Promega Corp., Madison, WI, USA). Renilla luciferase activity was used for normalization.

Transfection. miR-26b mimic and inhibitor were employed to overexpress or knockdown miR-26b (both from Guangzhou RiboBio Co., Ltd., Guangzhou, China). pcDNA3.1-JAG1 and its negative control (pcDNA3.1-NC) were designed and synthesized from Sangon Biotech Co., Ltd. (Shanghai, China). Human cervical cancer cells HeLa and JAR were seeded in 6-well plates and when the cells were 80% transfection was performed. pcDNA3.1-JAG1 and pcDNA3.1-NC were transfected with Lipofectamine 3000, whereas miR-26b mimic or inhibitor used Lipofectamine 2000 (both from Invitrogen; Thermo Fisher Scientific, Inc.).

Statistical analysis. The data were analyzed by Student's t-test and Pearson's χ^2 test using SPSS 19.0 software (IBM Corp., Armonk, NY, USA). One-way ANOVA, followed by Tukey's post hoc test, was employed to compare three or more groups. Survival was analyzed by Kaplan-Meier method with log rank test. The correlation between miR-26b and JAG1 was analyzed using Spearman's rank correlation coefficient. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-26b is downregulated in cervical cancer and is correlated with JAG1. The expression levels of miR-26b and JAG1 in cervical cancer tissues and cells were evaluated by RT-qPCR. miR-26b level was remarkably decreased in cervical cancer tissues compared with paracancerous tissues (P<0.001) (Fig. 1A). Moreover, in cervical cancer cells

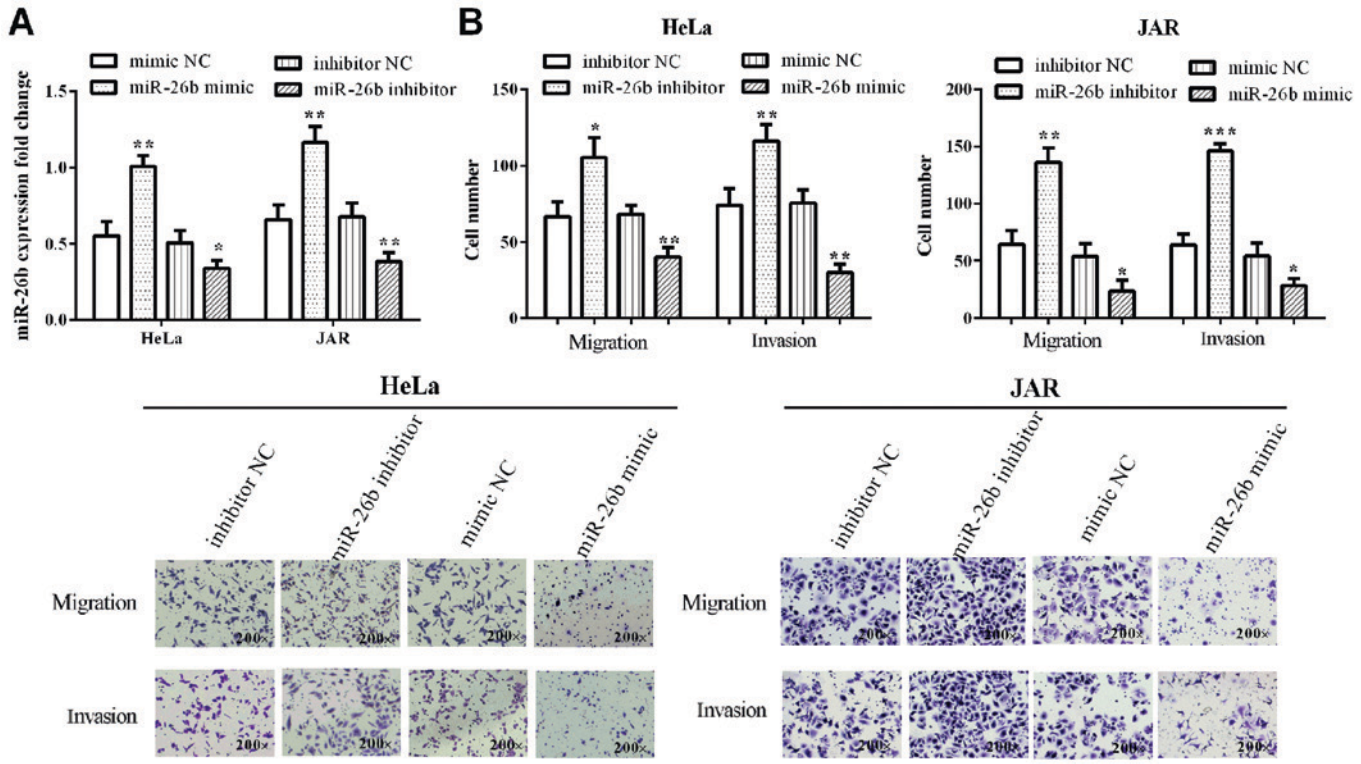

Figure 2. Influence of miR-26b on cervical cancer cell migration and invasion. (A) miR-26b expression was detected by RT-qPCR and (B) the migratory and invasive capacities were measured by Transwell assay, after transfection with miR-26b mimic or inhibitor. *P<0.05, **P<0.01, ***P<0.001. miR-26b, microRNA-26b.

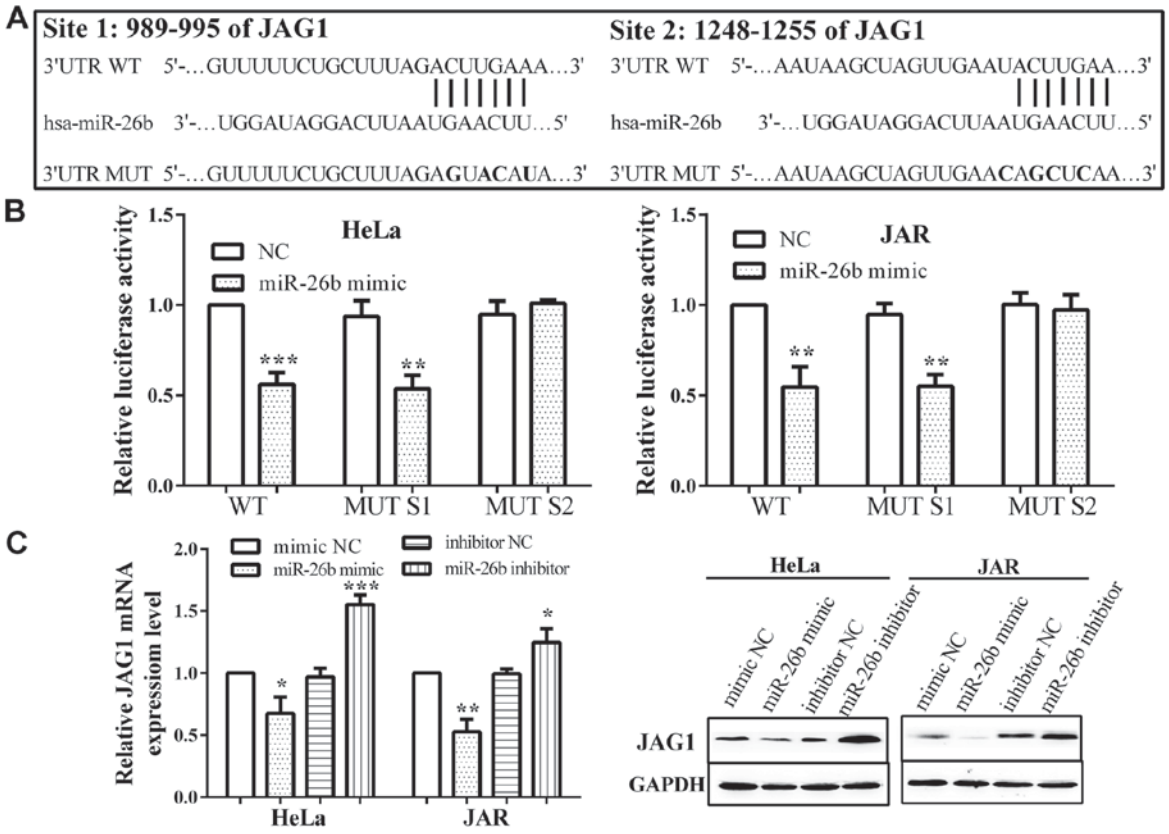

Figure 3. JAG1 was a target of miR-26b in cervical cancer cells. (A) Two binding sites of miR-26b on 3'UTR of JAG1 were predicted by TargetScan. (B) Changes in the luciferase activity of cells co-transfected with miR-26b mimic (or negative control) and WT or MUT S1/S2 in HeLa and JAR cells are presented. (C) The expression levels of JAG1 mRNA and protein were calculated by RT-qPCR and western blotting when transfected with miR-26b mimic or inhibitor. *P<0.05, **P<0.01, ***P<0.001. JAG1, Jagged1; miR-26b, microRNA-26b; 3'UTR, 3'-untranslated regions; WT, wild-type of JAG1 3'UTR; MUT S1/S2, mutant site 1/2 of JAG1 3'UTR.

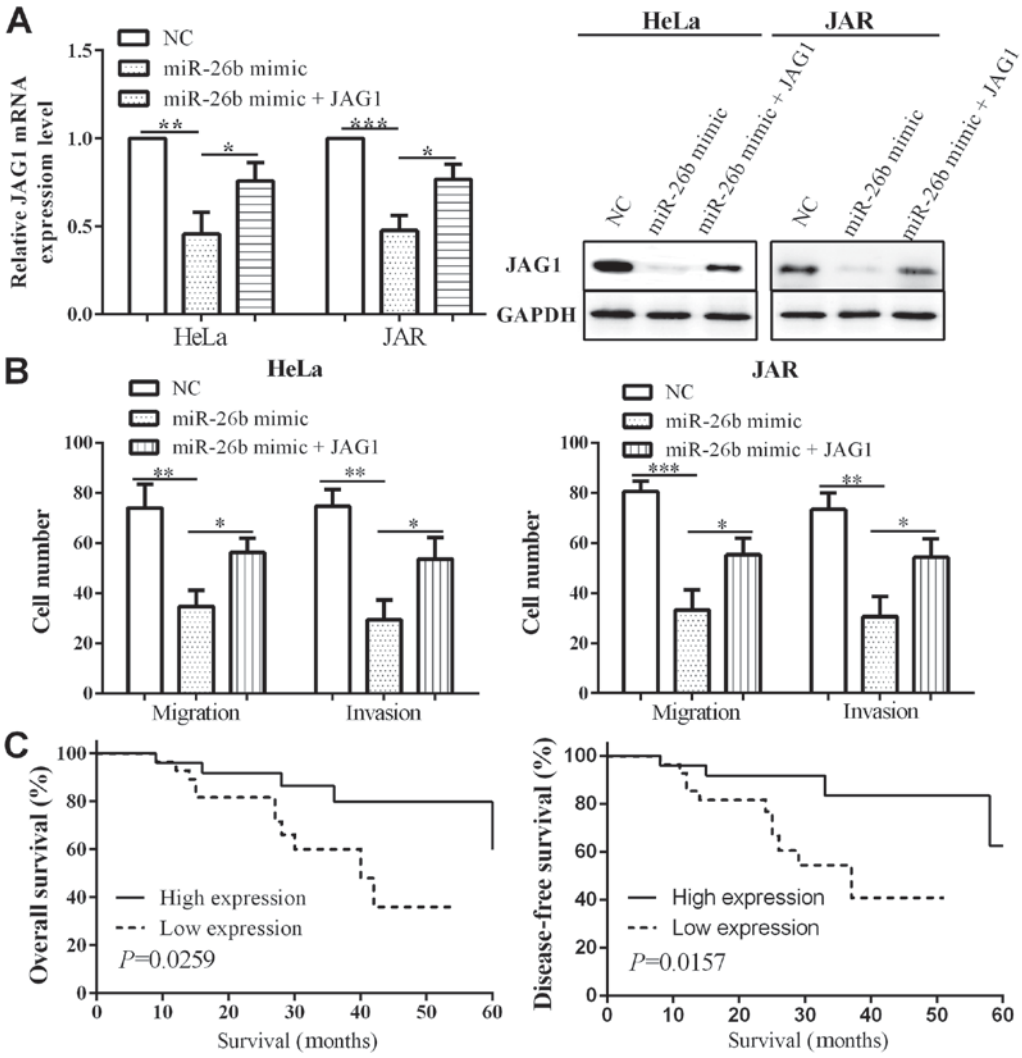

Figure 4. JAG1 restored partial impact of miR-26b in cervical cancer cells. (A) The expression levels of JAG1 were measured by RT-qPCR and western blotting when cervical cancer cells were transfected with miR-26b mimic or miR-26b mimic and JAG1 overexpression plasmid. (B) Migration and invasion were calculated when cervical cancer cells were transfected with miR-26b mimic or miR-26b mimic and JAG1. (C) Kaplan-Meier survival curves of cervical cancer patients with high or low miR-26b expression level. *P<0.05, **P<0.01, ***P<0.001. JAG1, Jagged1; miR-26b, microRNA-26b.

HeLa and JAR, miR-26b expression was lower than that in non-tumor epithelial Ect1/E6E7 cells (P=0.0026 and 0.0034, respectively) (Fig. 1B). The expression of JAG1, contrary to the expression of miR-26b, was overexpressed in cervical cancer HeLa (P=0.0008) and JAR (P=0.0012) cells versus Ect1/E6E7 (Fig. 1C). In addition, the expression of miR-26b and JAG1 were negatively correlated (P<0.0001, r=-0.5063) in cervical cancer tissues (Fig. 1D).

miR-26b expression suppresses cervical cancer migration and invasion. miR-26b mimic and inhibitor were transfected into cervical cancer HeLa (P=0.0026 and 0.040, respectively) and JAR cells (P=0.0038 and 0.0093, respectively) to overexpress or knock down miR-26b, which was measured by RT-qPCR (Fig. 2A). As predicted, in miR-26b mimic group cell migration and invasion were suppressed compared with negative control group in HeLa (P=0.0041 and 0.0015, respectively) and JAR (0.0233 and 0.0256, respectively) cells. Cell numbers were obviously increased in miR-26b inhibitor group compared with negative control group in HeLa (0.0144 and 0.0092) and JAR (0.0022 and 0.0003) cells (Fig. 2B). Thus, miR-26b suppresses cervical cancer cell migration and invasion.

miR-26b directly targets JAG1 and regulates JAG1 expression. TargetScan 4.2 (http://www.targetscan.org/vert_42/), a miRNA target identification tool, was utilized to search for potential target genes of miR-26b. We discovered that JAG1 is a potential target of miR-26b with two binding sites. The two binding sites were 5'-ACUUGAA-3' from 989 to 995 and from 1,248 to 1,255 on 3'UTR (Fig. 3A). Two miR-26b potential wild-types (WT) of JAG1 3'UTR and corresponding mutant sites (MUT S1/S2; 5'-AGUACAU-3' and 5'-AGCUCAA-3') were constructed and co-transfected with miR-26b mimic or negative control into HeLa and JAR cells to confirm miR-26b binding to JAG1. Luciferase activities were reduced in HeLa and JAR cells when transfected with miR-26b mimic in MUT S1 (P=0.0038 and 0.0016, respectively), compared with negative control. However, the luciferase activity was not influenced by miR-26b mimic in MUT S2 (P=0.2307 and 0.6472), while

it decreased in WT group (P=0.0003 and 0.0021), which reveals that miR-26b directly binds to site 2, rather than site 1 (Fig. 3B).

In addition, the mRNA (P=0.0132 and 0.0012) and protein level of JAG1 were reduced when overexpressed by miR-26b mimic in both HeLa and JAR cells. Also, miR-26b inhibitor could promote JAG1 expression (P=0.0006 and 0.0203) (Fig. 3C).

JAG1 partially reverses the impact of miR-26b. To further verify the miR-26b impact on cell migration and invasion via targeting JAR1, we detected JAG1 mRNA and protein level transfected with miR-26b and JAG1 in HeLa and JAR cells. As a result, when transfected with miR-26b mimic, the expression of JAG1 was reduced in both HeLa (P=0.0021) and JAR (P=0.0004) cells. When transfected with miR-26b mimic and JAG1, the mRNA and protein level of JAG1 (P=0.0425 and 0.0152) were increased, compared to transfection with only miR-26b mimic, which suggested that JAG1 could partially reverse the impact of miR-26b (Fig. 4A). When overexpressing miR-26b, the migration in HeLa and JAR cells (P=0.0043 and 0.0008, respectively) as well as invasion (P=0.0019 and 0.0022, respectively) were attenuated. Whereas, migration (P=0.0122 and 0.0232) and invasion (P=0.0251 and 0.0215) capacity increased when co-transfected with miR-26b mimic and JAG1, relative to transfection with miR-26b mimic alone (Fig. 4B). Thus, these results demonstrate that miR-26b inhibited cell migration and invasion by targeting JAG1 in cervical cancer.

miR-26b low expression predicts poor prognosis. Cervical cancer patients were segmented into different groups based on clinicopathological characteristics, in order to discover the relationship between miR-26b level and clinicopathological features of cervical cancer. It was found that miR-26b level was closely associated with FIGO stage (P=0.033), lymph node metastasis (P=0.025), serum SCC-Ag level (P=0.0327) and JAG1 level (P=0.030), while it had no association with age (P=0.860), tumor size (P=0.151), and histology degree (P=0.336) (Table I).

However, these clinicopathological factors can not be sufficient to accurately predict prognosis of patients. Patients were also set as miR-26b low expression group [miR-26b(-)] (n=28) if miR-26b expression was higher than the median value, and miR-26b high expression group [miR-26b(+)] (n=26) if miR-26b expression was higher than the median value. The overall survival (P=0.0259) and disease-free survival (P=0.0157) was obviously longer in miR-26b(+) group versus that of miR-26b(-) group (Fig. 4C).

Discussion

Cervical cancer is the second most frequent malignant neoplasm in women worldwide, and >80% are cervical squamous cell carcinomas (1). The exact reasons for cervical cancer occurrence are not fully understood (2). Therefore, exploring the biological mechanisms of cervical cancer metastasis and prognosis is necessary. In the present study, we demonstrated that miR-26b expression is obviously decreased in cervical cancer tissues, and is negatively correlated with JAG1. In addition, miR-26b affects cell proliferation by regulating JAG1, and patients with miR-26b low expression present poor overall and disease-free survival.

miRNAs, such as miR-365, miR-185, miR-23b, miR-133a and miR-26b (14,21-24), usually act as tumor suppressors. miR-26b, a member of miR-26 family, has been reported to be a tumor suppressor in oral squamous cell carcinoma, colorectal cancer, breast cancer and glioma (13-17). Fukumoto *et al* discovered that miR-26b inhibits cell proliferation, migration and invasion through targeting TMEM184B in oral squamous cell carcinoma (13). In breast cancer, miR-26b was found to suppress cell proliferation by targeting PTGS2 (15). Luo *et al* reported that miR-26b is low expressed in human cervical cancer and low-miR-26b expression predicts poor prognosis (25). Consistent with all the above findings, we found that miR-26b level is reduced in human cervical cancer tissues versus paracancerous tissues. Transfection of miR-26b mimics into HeLa and JAR cells causes cell migration and invasion reduction, thus for the first time it is proposed that miR-26b is involved in cervical cell migration and invasion. In addition, 54 patients were divided into high and low expression group, and the results revealed that the 5-year survival rate of low expression group was significantly lower than that of the high expression group, similarly to the findings of Luo *et al* (25).

It is well known that miRNAs play a crucial part in tumor development, proliferation, apoptosis and metastasis via regulating target gene expressions. miR-26b ectopic expression could inhibit glioma cell proliferation, migration and invasion via regulating EphA2 (17). Previous studies have reported that miR-26b mimics inhibit lens epithelial cell proliferation and EMT, and JAG1 has been identified as a direct target of miR-26b (19). However, in cervical cancer cells, there is little research on miR-26b mediation, and up to our knowledge we present for the first time that miR-26b impacts cervical cancer cell migration and invasion through targeting JAG1. In the present study, we found that JAG1 is a direct target of miR-26b with two binding sites on 3'UTR. To confirm targeting of JAG1 by miR-26b, luciferase reporter vector was constituted with a JAG1 3'UTR fragment containing the target sequence of miR-26b or a mutation fragment was inserted. Luciferase activities were found to decrease when transfected with WT and MUT S1, as opposed to MUT S2 vector, both in HeLa and JAR cells. Migration and invasion decreased when transfected with miR-26b mimic and this effect was partially reversed by transfection with JAG1. It has been reported that miR-26b level is correlated with FIGO stage, tumor size, lymph node metastasis and lymph-blood vessel invasion (26). Similarly, we found that miR-26b expression is associated with FIGO stage, lymph node metastasis, SCC-Ag and JAG1. Luo *et al* have reported that in human cervical cancer low-miR-26b expression predicts poor prognosis (25). In this study, patients with low miR-26b expression presented poor prognosis. Due to the small number of tissues, further research is needed of a larger population to ascertain the relationship between miR-26b expression and prognosis.

In conclusion, our study demonstrated that miR-26b affects cervical cancer cell migration and invasion through targeting JAG1, and may be a potential prognostic biomarker for cervical cancer patients.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

LW was involved in the conception of the study and contributed in the writing of the manuscript; WW acquired the data and assisted with the data analyses; YW contributed significantly in the data analyses and assisted in the interpretation of the data with constructive discussions. All authors read and approved the final manuscript.

Ethics approval and consent to participate

For all the specimens informed consent was obtained from the patients and the study was approved by the Ethics Committee of Shangluo Central Hospital (Shangluo, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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