RESEARCH ARTICLE

In vivo and in vitro effects of microRNA-124 on human gastric cancer by targeting JAG1 through the Notch signaling pathway

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Funding information
The National Natural Science Foundation of China, Grant numbers: 81303135, 81573749; the Basic Science Research in Shaanxi Province of China, Grant number: 2015JQ8288

Abstract
In this study, we aim to determine the function of miR-124 on gastric cancer (GC) cells and the underlying mechanism that involves jaddeg1 (JAG1) and the Notch signaling pathway. GC tissues and adjacent tissues from 100 patients suffering from GC were selected. GC SGC-7901 and AGS cells were selected and grouped into control, mimic-NC, miR-124 mimic, inhibitor-NC, miR-124 inhibitor, and miR-124 inhibitor + si-JAG1 groups. RT-qPCR and a Western blotting assay were conducted to detect the expression of miR-124, JAG1, NICD, HES1, and HES5. MTS, wound-healing, transwell assay and flow cytometry were performed to detect cell proliferation, migration, invasion, cell cycle distribution, and apoptosis, respectively. Compared with adjacent tissues, a lower miR-124 expression and higher JAG1 expression were found in GC tissues. JAG1 is a direct target gene of miR-124. Compared with the control group, the expression of JAG1, NICD, HES1, and HES5, cell invasion, migration, and proliferation in the miR-124 mimic group were decreased, while the apoptosis rate was increased and cells were arrested at the G0/G1 phase. Compared with the miR-124 inhibitor group, the expression of JAG1, NICD, HES1, and HES5, cell invasion, migration, and proliferation in the miR-124 mimic + si-JAG1 group were decreased, while the apoptosis rate and cell ratio at the G0/G1 phase were increased. The demonstration that miR-124 inhibits GC cell growth supports the concept that miR-124 functions as a tumor suppressor by a mechanism that involves translational repression of the JAG1 and the inhibition of Notch signaling pathway.

KEYWORDS
apoptosis, gastric cancer, invasion, jagged1, microRNA-124-3p, migration, notch signaling pathway, proliferation

1 | INTRODUCTION

Gastric cancer (GC) is a pathophysiologically heterogeneous disease related to predominantly lymphatic spread, intrabdominal spread, or hematogenous metastasis. Some risk factors for GC include excessive alcohol, unbalanced diet, Helicobacter pylori infections, and host genetic diversity. In spite of the decreased GC incidence and mortality rates and numerous advancements in the treatment of GC, it still remains to be one of the most common malignant diseases and...
the third leading cause for cancer-related deaths across the world.\textsuperscript{3,4} The complete understanding of the underlying molecular mechanisms of tumor progression is beneficial to find novel paradigms for GC diagnosis and therapy.\textsuperscript{5} Previous studies proved that microRNAs (miRNAs) and their target genes play a vital role in many biological processes like cell development, proliferation, migration, invasion, apoptosis, and differentiation.\textsuperscript{6–8} Therefore, it is critical to investigate the effects of miRNAs on the progression of GC.

miRNAs are small non-coding RNAs, 21-25 nucleotide-long, which influence the expression of proteins by incompletely complementing the 3′-untranslated region (3′-UTR) of target genes.\textsuperscript{7,8} Hsa-miR-124-3p (miR-124), a kind of miRNAs, plays a crucial role in brain development and neuronal differentiation.\textsuperscript{9} Some studies demonstrated that down-regulated miR-124 expression was found in various cancers, including GC and breast cancer.\textsuperscript{10,11} In GC, decreased miR-124 expression is related to the differentiation degree, clinical disease stage, and lymph node metastasis.\textsuperscript{12} Moreover, miR-124 was proved to inhibit GC cell proliferation but promote apoptosis by targeting \textit{EZH2} and \textit{ROCK1} genes.\textsuperscript{13,14} Acting as a Notch ligand, \textit{jagged1} (JAG1) is widely expressed in mammals across multiple tissues and developing stage and is essential to various signaling pathways.\textsuperscript{15,16} Additionally, it has been shown that the Notch signaling regulates both development and cell responses to extracellular insults and has a cross-talk with miRNAs.\textsuperscript{17,18} From all that mentioned above, it was suggested that the Notch signaling pathway is essential to various cellular responses in GC. Therefore, we explored the effects of miR-124 on GC by targeting JAG1 through the Notch signaling pathway.

2 MATERIALS AND METHODS

2.1 Ethics statement

This study was approved by the Ethics Committee of Affiliated Hospital of Shaanxi University of Chinese Medicine and Department of Hepatobiliary Surgery of Xianyang Central Hospital, and signed informed consents were obtained from all patients participating in the study.

2.2 Study subjects

From January 2015 to January 2017, a total of 100 GC patients (66 males and 34 females, aged between 39 and 70 years, calculated mean age 53.2 ± 1.9 years) were selected at the Department of Surgical Oncology of Affiliated Hospital of Shaanxi University of Chinese Medicine and Department of Hepatobiliary Surgery of Xianyang Central Hospital for the collection of GC and adjacent tissues. None of patients included in the study underwent chemotherapy or radiotherapy prior to surgery. According to the World Health Organization (WHO) histological classification and criteria of GC,\textsuperscript{19} there were a total of 22 cases of high differentiation, 25 cases of middle differentiation, and 53 cases of low differentiation. There were 43 cases of lymph node metastasis and 57 cases without lymph node metastasis. In addition, there were 3 cases at the clinical stage I, 9 cases at the clinical stage II, 32 cases at the clinical stage III, and 56 cases at the clinical stage IV. There were 39 cases with serosal infiltration and 61 cases without serosal infiltration. GC tissue and adjacent tissue samples were promptly stored in a frozen tube and kept in a liquid nitrogen tank for preservation for further use.

2.3 Cell culture

Human normal gastric epithelial cells (GES-1) and GC cells (MKN-28, MGC-803, SGC-7901, AGS, and MKN-45) purchased from American Type Culture Collection (ATCC) were incubated in at 37°C in humidified incubator (Thermo Fisher Scientific, San Jose, CA) containing 5% CO\textsubscript{2} in air. The incubator was supplemented with RPMI 1640 culture medium (Hyclone Company, Logan, UT) containing 10% fetal bovine serum (FBS) (Gibco Company, Grand Island, NY) and 100 units/mL penicillin-streptomycin (Gibco Company). MiR-124 expression in cells was detected, and the GC cells presenting with the most different miR-124 expression compared to the GES-1 cell were selected for further experimentation. The cells were incubated under conventional conditions. The follow-up experiments were performed with 0.25% trypsin (Gibco Company) digestion and passage when cells were covered with 80% microscopic view.

2.4 Cell transfection and grouping

Oligonucleotide fragments miR-124 mimic, mimic-NC (negative control oligonucleotides), miR-124 inhibitor, inhibitor-NC, and si-JAG1 for transfection were all purchased from Shanghai GenePharma Co., Ltd (Shanghai, China). Cells were transfected and divided into the following six groups: the control, mimic-NC, miR-124 mimic, inhibitor-NC, miR-124 inhibitor, and miR-124 inhibitor + si-JAG1 groups. Cells were inculcated in the plate and incubated under conventional conditions 24 h prior to transfection. The culture medium was changed 1 h before transfection, and 2 mL conventional culture medium was added to each well. The mixture for transfection was prepared in accordance with instructions of the Lipofectamine 2000 kit (Invitrogen, Inc., Carlsbad, CA). Serum-free and double antibody-free mediums were added to the control group. Serum-free and double antibody-free mediums containing the corresponding oligonucleotide fragments (all final concentration was 300 pmol/well) covered by liposome (Invitrogen, Inc.) were added to the experimental group. Transfected cells were incubated in a serum-free medium for 4 h, and later, 10% fetal
bovine serum (FBS) was added. Then cells were placed in a humidified incubator at 37°C containing 5% CO₂ in air.

2.5 | Dual-luciferase reporter gene assay

Online bioinformatics prediction software TargetScan was employed in order to detect the binding sites of miR-124 and JAG1. JAG1 3′-UTR fragments lacking the miR-124 binding sites were inserted into the pMIR-REPORT vector (Ambion, Carlsbad, CA). Subsequently, the wild-type plasmid pMIR-JAG1-3′-UTR-wt (JAG1-wt) and the mutant plasmid pMIR-JAG1-3′-UTR-mut (JAG1-mut) were constructed. A 96-well plate (4 × 10⁴ cells/well with four duplicated wells) was used to inoculate SGC-7901 and AGS cells 24 h before transfection. The culture medium was changed 1 h before transfection and conventional culture medium was added into each well. The mixture for transfection was prepared according to instructions of the Lipofectamine 2000 kit (11668-027, Invitrogen, Inc.) and was added into the plate after culture medium was absorbed. A dual-luciferase reporter gene assay kit (Promega Corporation, Madison, WI) was conducted in order to detect transcriptional activity 24 h after incubation at 37°C. Co-transfection groups were as follows: miR-124 mimic + JAG1-wt, mimic-NC + JAG1-wt, miR-124 mimic + JAG1-mut, mimic-NC + JAG1-mut. All experiments were performed in triplicates.

2.6 | Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from cancer tissues, adjacent tissues, and cells in each groups using Trizol (15596026, Invitrogen Inc.) in accordance with the instructions of miRNeasy Mini kit (217004, Qiagen Company, Hilden, Germany). The RNA purity and concentration were measured by Nanodrop 2000 (Thermo Fisher Scientific) and subsequently the RNA were stored at −80°C. The special stem-loop primer RT primer (0.15 μm) was used to synthesize cDNA of miR-124 and U6 (Table 1). On the basis of gene sequences published in GenBank database, Primer 5.0 primer design software was applied in order to design PCR reaction primers (Table 1) synthesized by Shanghai GenePharma Co., Ltd. ABI PRISM 7500 real-time PCR System (ABI Company, Oyster Bay, NY) and SYBR Green I PCR Master Mix (Takara Holdings, Inc., Kyoto, Japan) were performed in PCR reaction. U6 snRNA/glyceraldehyde-3-phosphate dehydrogenase (U6/GAPDH) was used as the internal control. Dissolution curve was conducted to verify the reliability of the achieved PCR results. Cycle threshold value (Ct) was selected and served as the inflection point on the amplification power curve, ΔCt = CT (target gene), −CT (internal control), ΔΔCt = ΔCt (the experimental group), −ΔCt (the control group). The relative expression of target gene was calculated as 2−ΔΔCt. All experiments were performed in triplicates.

2.7 | Western blotting assay

Total proteins of tissues and cells were extracted, and protein concentrations were measured according to instructions of bicinchoninic acid (BCA) kit (Bio-Rad, Hercules, CA). The extracted proteins were added to the sample buffer solution (30 μg/per well) and then heated at 95°C for 10 min. Ten percent polyacrylamide gel electrophoresis (PAGE) (Bio-Rad) was employed in order to separate proteins with electrophoresis voltages from 80-120 V and wet transfer was conducted with 100 mV transmembrane voltage (45-70 min). The polyvinylidene fluoride (PVDF) transmembrane was conducted and the proteins were blocked using 5% bovine serum albumin (BSA) for 1 h at room temperature. The primary antibodies (diluted at a ratio of 1:1000) were added at 4°C for overnight: JAG1 (ab7771, Abcam, Inc., Cambridge, MA), Caspases 3 (ab32042, Abcam, Inc.), Bax (ab32503, Abcam, Inc.), NICD (ab8925, Abcam, Inc.), Bax (ab32503, Abcam, Inc.), NIDC (ab8925, Abcam, Inc.).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5′-3′)</th>
</tr>
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<tbody>
<tr>
<td>miR-124</td>
<td>RT Primer: GTCGTATCCAGTGCAAGGGTCGGTGATTCGACTGGATACGACGGCACTTC</td>
</tr>
<tr>
<td>U6</td>
<td>RT Primer: GTCGTATCCAGTGCAAGGGTCGGTGATTCGACTGGATACGACGGCACTTC</td>
</tr>
<tr>
<td>miR-124</td>
<td>Forward: ATGGTTGTTGGTAAAGGCACCCGG</td>
</tr>
<tr>
<td></td>
<td>Reverse: GCAGGCTCCGAGGTATTC</td>
</tr>
<tr>
<td>U6</td>
<td>Forward: TCCGTCTGCCAGCACATATAC</td>
</tr>
<tr>
<td></td>
<td>Reverse: TATGGGAGGCTCTTGAGGATATTC</td>
</tr>
<tr>
<td>JAG1</td>
<td>Forward: GGGGCAACACCTTCAACCTC</td>
</tr>
<tr>
<td></td>
<td>Reverse: CACAGCCCTCCACACGCAATC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: ACGGATTTTGGTGCGATTGGG</td>
</tr>
<tr>
<td></td>
<td>Reverse: TCGCTCTGGAAGATGGTGAT</td>
</tr>
</tbody>
</table>

miR-124, hsa-miR-124-3; U6, U6 snRNA; JAG1, jagged1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-qPCR, reverse transcription quantitative polymerase chain reaction.
Inc., HES1 (ab71559, Abcam, Inc.), HES5 (ab25374, Abcam, Inc.), and β-actin (ab8226, Abcam, Inc.). Samples were rinsed with tris-buffered saline tween 20 (TBST) (5 min/three times). The secondary antibody horse radish peroxidase (HRP) was added to samples and incubated at room temperature for 1 h. After fully membrane rising (5 min/three times), samples were developed using chemiluminescence reagent and β-actin was used as the internal control. HRP was developed by electro-chemiluminescence (ECL), scanned and recorded by Bio-Rad Gel Dol EZ Imager (Bio-Rad Laboratories, Inc.). Gray value analysis of the target banding was conducted using ImageJ software. All experiments were performed in triplicates.

2.8 MTS assay

The transfected cells were seeded in a 96-well plate upon dilution to designed concentrations (5 × 10³ cells/well). Cells were incubated in CO₂ for 24 h, 48 h, and 72 h at 37°C. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt, G3582, Promega Corp.) of 20 μL was added to each well and the incubation was continued for another 4 h. The corresponding optical density (OD) was measured at a wavelength of 490 nM per well using a multi-mode microplate reader (Molecular Devices, Sunnyvale, CA). Then, survival rates of GC cells were calculated based on the following formula: cell survival rate (%) = OD value the experimental group/OD value the control group × 100%. All experiments were performed in triplicates.

2.9 Wound-healing assay

Cells were seeded in a six-well plate and incubated at 37°C post-transfection. The cells completely adhered to the bottom plate, and a line was drawn in each well using a sterilized gun head with consistent width. The cells were photographed, and the six-well plate was marked in order to locate in the same vision field conveniently. This time was as 0 h, and cell culture medium was taken out 24 h after cell incubation at 37°C. The six-well plate was rinsed with PBS three times until cell debris caused by wound-healing was washed away, and a serum-free medium was added. Photographs were taken by Olympus inverted microscope (Olympus Optical Co., Ltd., Tokyo, Japan) and time was as 24 h at that juncture. Six visions with fixed position were selected and cells migrating into the scratch area were counted using the click-counting software. All experiments were performed in triplicates.

2.10 Transwell assay

Matrigel (3.9 mg/mL, 60-80 μL) was added to the Transwell chamber (140629, Thermo Fisher Scientific, Inc., Roskilde, Denmark) and they were incubated at 37°C for 30 min until Matrigel was attained a concrete structure. Pre-warmed culture medium was added to the cell culture plate and hydrated for 2 h in a cell incubator at 37°C. The 0.5 mL cell suspensions (5 × 10⁴ cells/mL) were added to Transwell chamber and incubation was conducted at 37°C for 24 h. After the chamber was taken out, cells in upper chamber were rinsed with PBS and soaked in pre-cooled methanol. Then, cells migrating into the lower chamber were fixed and stained with 0.1% crystal violet solution for 10 min. An Olympus inverted microscope was applied to observe cells and photos were taken. Six visions with fixed position were selected and cells migrating into the scratch area were counted using the click-counting software. All experiments were performed in triplicates.

2.11 Flow cytometry

Trypsin was used in order to digest and centrifuge cells and cells then were collected 24 h post-transfection. Cells were rinsed with cold PBS and re-suspended with PBS buffer solution (containing calcium) into a single cell suspension (1 × 10⁶/mL). The tube was filled with 100 μL cell suspension at room temperature, and propidium iodide (PI) (Sigma-Aldrich Chemical Company, Louis, MO) and RNase A were mixed with final concentration of 10 mg/mL. Cells were incubated at 4°C for 30 min and 400 μL staining buffer were added. Immediately, flow cytometry (BD and Company Bioscience, San Jose, CA) was performed in order to analyze and detect and 10⁴ cells were detected each time. The Cell Quest software was used for data analysis. Annexin V-positive cells served as apoptotic cells. PI-positive and Annexin V-negative cells served as necrotic cells. Apoptosis rate = (Annexin V⁺PI⁺ cell numbers + Annexin V⁺PI⁻ cell numbers)/10⁴ × 100%). For cell cycle detection, a tube was filled with 100 μL cell suspension and 1 mL PI/TritonX-100 staining solution (containing 0.2 mg RNase A, 20 μg PI, and 0.1% TritonX-100) was added upon fully mixing. Cells were incubated for 30 min at 4°C and then flow cytometry was conducted to detect cell cycle. All experiments were performed in triplicates.

2.12 Statistical analysis

All experiments were repeated three times. Statistical analyses were performed using the SPSS 21.0 software (IBM Corp., Armonk, NY). Measurement data were presented as mean ± standard deviation (SD). Differences between two groups were analyzed using the t-test, and statistical analysis among multiple groups was conducted using the one-way analysis of variance (ANOVA). Homogeneity of variance was conducted using variance analysis, while heterogeneity of variance was conducted using the Wilcoxon rank sum test. Correlations between gene expression and clinical cases were analyzed by Chi-square test and Fisher's exact test. P < 0.05 was considered to be statistically significant.
3 | RESULTS

3.1 | Lower miR-124 expression and higher JAG1 expression in GC tissues

We previously found miR-124 interacts with the Notch1 signaling pathway and has therapeutic potential against GC. Also, the activation of JAG1-Notch1 signaling is significantly associated with acquired chemoresistance in cancers. In order to investigate the roles of miR-124 and JAG1 in GC, RT-qPCR, and a Western blotting assay were firstly conducted to detect the expression of miR-124 and JAG1 in GC tissues and adjacent tissues. Lower miR-124 expressions but higher JAG1 mRNA expressions were found in GC tissues than in adjacent tissues (all \(P < 0.05\), Figures 1A and 1B). Moreover, JAG1 protein expressions also showed higher expression in GC tissues in compared to adjacent tissues (all \(P < 0.05\), Figures 1C and 1D). In addition, negative correlations were found between miR-124 and JAG1 expression by Pearson’s correlation analysis (Figure 1E).

FIGURE 1  Expression of miR-124 and JAG1 in GC tissues and adjacent tissues (n = 100). A, expression of miR-124 in GC tissues and adjacent tissues detected by RT-qPCR; B, mRNA expression of JAG1 in GC tissues and adjacent tissues detected by RT-qPCR; C, protein expression of JAG1 in GC tissues and adjacent tissues detected by Western blotting assay; D, gray values of protein expression in panel C; E, the negative association between miR-124 and JAG1 by Pearson’s correlation analysis. t-test was applied for statistical analysis; *, \(P < 0.05\); miR-124, hsa-miR-124-3p; JAG1, jagged1; GC, gastric cancer; RT-qPCR, reverse transcription quantitative polymerase chain reaction; NC, negative control.
shown between miR-124 and JAG1 mRNA expressions ($R = -0.76, P < 0.001$) (Figure 1E).

### 3.2 miR-124 and JAG1 expressions are associated with clinicopathological features of GC patients

Subsequently, to analyze the correlations of miR-124 and JAG1 mRNA expression with clinicopathological features, the relative expression of miR-124 and JAG1 were obtained according to RT-qPCR. Next, their expression among the different clinical pathologies were observed and analyzed. The results indicated that, there were significant associations of miR-124 expressions with differentiation degree, lymph node metastasis, clinical stage and infiltration degree of GC patients, but no correlations with age and gender. In details, compared with the well differentiated samples, a significantly decreased miR-124 expression was observed in the poorly differentiated samples ($P < 0.05$). A lower expression of miR-124 was observed in the samples with lymph node metastasis than the samples without lymph node metastasis ($P < 0.05$). MiR-124 expressions were down-regulated in samples at stages II, III, and IV instead of samples at stage I ($P < 0.05$) (Table 2). On the other hand, in contrast to the well differentiated samples, samples without lymph node metastasis, and samples at stage I, JAG1 showed increased mRNA expressions in poorly differentiated samples, samples with lymph node metastasis, and samples at stages II, III, and IV (all $P < 0.05$) (Table 2). However, no correlations were found between JAG1 mRNA expressions and age (or gender) (Table 2). All above results implied that miR-124 and JAG1 might play vital roles in the development of GC.

### 3.3 Lower expression of miR-124 and higher expression of JAG1 in GC cells

To study the mechanisms and functions of miR-124 and JAG1 in GC, their expression in gastric epithelial cell GES-1 and GC cell lines including MKN-28, MGC-803, SGC-7901, AGS, and MKN-45 GC cells were determined using RT-qPCR and a Western blot assay. The results showed that GES-1 cells exhibited a higher miR-124 expression than other GC cells, among which the lowest miR-124 expression was found in SGC-7901 and AGS cells. However, an opposite trend was observed in the expression of JAG1 and miR-124. The lowest expression of JAG1 was observed in the GES-1 cells, and the

<p>| TABLE 2 | Correlations of miR-124 and JAG1 expressions with clinicopathological features of patients with gastric cancer |</p>
<table>
<thead>
<tr>
<th>Factor</th>
<th>Case</th>
<th>miR-124 relative expression</th>
<th>$P$</th>
<th>JAG1 relative expression</th>
<th>$P$</th>
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<td>Age</td>
<td></td>
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<tr>
<td>≤52.3</td>
<td>35</td>
<td>0.58 ± 0.11</td>
<td>0.535</td>
<td>2.12 ± 0.36</td>
<td>0.824</td>
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<tr>
<td>&gt;52.3</td>
<td>65</td>
<td>0.59 ± 0.15</td>
<td>0.346</td>
<td>2.14 ± 0.46</td>
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<tr>
<td>Gender</td>
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<tr>
<td>Male</td>
<td>66</td>
<td>0.60 ± 0.15</td>
<td>&lt;0.001</td>
<td>2.10 ± 0.44</td>
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<tr>
<td>Female</td>
<td>34</td>
<td>0.56 ± 0.12</td>
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<td>2.19 ± 0.40</td>
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<tr>
<td>Differentiation</td>
<td></td>
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<tr>
<td>Well differentiation</td>
<td>22</td>
<td>0.70 ± 0.17</td>
<td>&lt;0.001</td>
<td>1.72 ± 0.52</td>
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<tr>
<td>Moderate differentiation</td>
<td>25</td>
<td>0.61 ± 0.13</td>
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<td>2.18 ± 0.39</td>
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<tr>
<td>Poor differentiation</td>
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<td>0.53 ± 0.09</td>
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<td>2.28 ± 0.27</td>
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<td>Lymph node metastasis</td>
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<td>0.001</td>
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<td>&lt;0.001</td>
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<tr>
<td>Yes</td>
<td>43</td>
<td>0.54 ± 0.08</td>
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<td>2.31 ± 0.22</td>
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</tr>
<tr>
<td>No</td>
<td>57</td>
<td>0.63 ± 0.16</td>
<td>&lt;0.001</td>
<td>2.00 ± 0.49</td>
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<tr>
<td>Clinical stage</td>
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<tr>
<td>I</td>
<td>3</td>
<td>0.90 ± 0.05</td>
<td>&lt;0.001</td>
<td>1.22 ± 0.07</td>
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<td>II</td>
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<tr>
<td>III</td>
<td>32</td>
<td>0.62 ± 0.14</td>
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<td>2.04 ± 0.40</td>
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<tr>
<td>IV</td>
<td>56</td>
<td>0.56 ± 0.13</td>
<td>&lt;0.001</td>
<td>2.26 ± 0.38</td>
<td>&lt;0.001</td>
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<td>Infiltration degree</td>
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<tr>
<td>Serosal invasion</td>
<td>61</td>
<td>0.64 ± 0.14</td>
<td>&lt;0.001</td>
<td>1.92 ± 0.41</td>
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<tr>
<td>Without serosal invasion</td>
<td>39</td>
<td>0.50 ± 0.07</td>
<td></td>
<td>2.46 ± 0.18</td>
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</table>

$t$-test and one-way ANOVA were used for statistical analysis.

miR-124, hsa-miR-124-3; JAG1, jagged1.
highest JAG1 expression was observed in the SGC-7901 and AGS cells. The differences were statistically significant (all \( P < 0.05 \)) (Figure 2). The expression of miR-124 was significantly different among the GES-1, SGC-7901, and AGS cells, and therefore the SGC-7901 and AGS cells were selected for the follow-up experiments.

### 3.4 miR-124 directly targets JAG1

Online bioinformatics prediction software TargetScan was employed in order to verify that JAG1 was the downstream target gene of miR-124 (Figure 3A). SGC-7901 and AGS cells were used for experiments. Based on the results of dual-luciferase reporter gene assay, the miR-124 mimic group showed significant inhibition on the luciferase activity of JAG1-3′UTR-wt, but no obvious inhibition on the luciferase activity of JAG1-3′UTR-mut (all \( P < 0.05 \)) (Figures 3B and 3C), demonstrating that JAG1 is a direct target gene of miR-124.

### 3.5 Regulatory effects of miR-124 on JAG1 in GC cells

Next, the detailed regulatory effects of miR-124 on JAG1 were studied in SGC-7901 and AGS. The results indicated that, compared with the mimic-NC and control groups, the expression of miR-124 was increased in the miR-124 mimic group while the expression of JAG1 was evidently decreased. Compared with the inhibitor-NC and control groups, the expression of miR-124 in the miR-124 inhibitor and miR-124 inhibitor + si-JAG1 groups was decreased, while the expression
**FIGURE 3** JAG1 is a direct target gene of miR-124 by dual-luciferase reporter gene assay. A, binding sites of miR-124 and JAG1 predicted by public database; B, miR-124 negatively regulated JAG1 expression in SGC-7901 cells by dual-luciferase reporter gene assay; C, miR-124 negatively regulated JAG1 expression in AGS cells by dual-luciferase reporter gene assay. Each group had three samples, and each sample was repeated three times. *t*-test was used for statistical analysis; *, *P* < 0.05, compared with the control group; miR-124, hsa-miR-124-3p; NC, negative control; JAG1, jagged1

**FIGURE 4** Comparisons of expression of miR-124 and JAG1 in transfected cells among six groups. A, expression of miR-124 and JAG1 mRNA in SGC-7901 cells by RT-qPCR; B, expression of JAG1 protein in SGC-7901 cells detected by Western blotting assay; C, gray value analysis of nine Western plots of JAG1 protein densities in (C) by Western blotting assay; D, expression of miR-124 and JAG1 mRNA in AGS cells by RT-qPCR; E, expression of JAG1 protein in AGS cells detected by Western blotting assay; F, gray value analysis of nine Western plots of JAG1 protein densities in panel G by Western blotting assay. Each group had three samples, and each sample was repeated three times. One-way ANOVA was used for statistical analysis; *, *P* < 0.05, compared with the control group; #, *P* < 0.05, compared with the mimic-NC group; Δ, *P* < 0.05, compared with the inhibitor-NC group; &, *P* < 0.05, compared with the miR-124 inhibitor group; miR-124, hsa-miR-124-3p; JAG1, jagged1; GC, gastric cancer; RT-qPCR, quantitative real-time polymerase chain reaction; NC, negative control
of JAG1 was significantly increased. Besides, the expression of JAG1 in the miR-124 inhibitor + si-JAG1 group was downregulated in comparison with the miR-124 inhibitor group (all \( P < 0.05 \)) (Figure 4). The results verified that miR-124 mimic could increase the expression of miR-124, but reduce the expression of JAG1 in the SGC-7901 and AGS cells, while the miR-124 inhibitor showed opposite results in SGC-7901 and AGS cells. Besides, the similar conclusions obtained from two GC cells suggested the universality.

3.6 miR-124 inhibits the Notch signaling pathway in GC cells

Considering that JAG1 is the key ligand for Notch, the expression of the Notch signaling pathway-related proteins in SGC-7901 and AGS cells was tested. The results demonstrated that there were no significant expression differences of Notch signaling pathway-related proteins (NICD, HES1, and HES5) among the control, mimic-NC, inhibitor-NC, miR-124 inhibitor + si-JAG1 groups (all \( P > 0.05 \)). Also, in contrast to the mimic-NC and control groups, the expression of NICD, HES1, and HES5 in the miR-124 mimic group was decreased. In comparison with the inhibitor-NC and control groups, the expression of NICD, HES1, and HES5 in the miR-124 inhibitor group was increased. Besides, compared with the miR-124 inhibitor group, the expression of NICD, HES1, and HES5 in the miR-124 inhibitor + si-JAG1 group was downregulated (all \( P < 0.05 \)) (Figure 5). The results indicated that miR-124 mimic could decrease the expression of Notch signaling pathway-related proteins (NICD, HES1, and HES5), but miR-124 inhibitor could increase their expressions in SGC-7901 and AGS cells.

**FIGURE 5** miR-124 mediates the Notch signaling pathway-related proteins (NICD, HES1, and HES5) among six groups. A, expression of the Notch signaling pathway-related proteins in SGC-7901 cells detected by Western blotting assay; B, gray value analysis of nine Western plots of protein expression in panel A; C, expression of the Notch signaling pathway-related proteins in AGS cells by Western blotting assay; B, gray value analysis of nine Western plots of protein expression in panel C. Each group had three samples, and each sample was repeated three times. One-way ANOVA was used for statistical analysis; *, \( P < 0.05 \) compared with the control group, #, \( P < 0.05 \) compared with the mimic-NC group; Δ, \( P < 0.05 \) compared with the inhibitor-NC group; &, \( P < 0.05 \) compared with the miR-124 inhibitor group; miR-124, hsa-miR-124-3p; NC, negative control.
3.7 | miR-124 inhibits GC cell proliferation

In the following experiments, we mainly investigated the effects of miR-124 on the biological functions of GC cells. The MTS assay showed that, there was no evident difference concerning cell proliferation among the control, mimic-NC, inhibitor-NC, and miR-124 inhibitor + si-JAG1 groups (all \( P > 0.05 \)). Compared with the mimic-NC and control groups, GC cell proliferation in the miR-124 mimic group was evidently inhibited (all \( P < 0.05 \)). In contrast to the inhibitor-NC and control groups, an increased GC cell proliferation was observed in the miR-124 inhibitor group (all \( P < 0.05 \)). Compared with the miR-124 inhibitor group, GC cell proliferation in the miR-124 inhibitor + si-JAG1 group was decreased (both \( P < 0.05 \)) (Figure 6). The results indicated that miR-124 inhibited the proliferation of GC cells.

3.8 | miR-124 suppresses GC cell invasion and migration

Additionally, the effects of miR-124 on invasion and migration of SGC-7901 and AGS cells were detected using wound-healing and transwell assays. Cell migration and invasion abilities showed no difference in the control, mimic-NC, inhibitor-NC, and miR-124 inhibitor + si-JAG1 groups (all \( P > 0.05 \)). Compared with the mimic-NC and control groups, an increased GC cell proliferation was observed in the miR-124 inhibitor group (all \( P < 0.05 \)). Compared with the miR-124 inhibitor group, GC cell proliferation in the miR-124 inhibitor + si-JAG1 group was decreased (both \( P < 0.05 \)) (Figure 6). The results indicated that miR-124 could suppress the invasion and migration abilities of GC cells.

3.9 | miR-124 reduces GC cell cycle progression

Subsequently, the effects of miR-124 on the cell cycle distribution of GC cells were studied. GC cells (SGC-7901 and AGS) were incubated for 24 h post-transfection, and flow cytometry was conducted in order to detect cell cycle distribution. There were no obvious differences of cell cycle stages in the control, mimic-NC, inhibitor-NC and miR-124 inhibitor + si-JAG1 groups (all \( P > 0.05 \)). In contrast to the mimic-NC and control groups, the miR-124 mimic group showed significantly increased ratio of cells at the G0/G1 phase of the cell cycle and decreased ratio of cells at the G2/M and S phases of the cell cycle. Compared with the inhibitor-NC and control groups, the miR-124 inhibitor group had lower ratio of cells at the G0/G1 phase of the cell cycle and higher ratio of cells at the G2/M and S phases of the cell cycle. The miR-124 inhibitor + si-JAG1 group showed significantly more cells at the G0/G1 phase of the cell cycle but less cells at the G2/M and S phases of the cell cycle compared to the miR-124 inhibitor group (both \( P < 0.05 \)) (Table 3). The results revealed that miR-124 regulated cell cycle distribution by arresting GC cells at the G0/G1 phase.

3.10 | miR-124 facilitates GC cell apoptosis

Lastly, flow cytometry was conducted in order to detect the effects of miR-124 on the apoptosis of SGC-7901 and AGS cells. No cell apoptosis differences were observed among the control, mimic-NC, inhibitor-NC, and miR-124 inhibitor + si-JAG1 groups.
miR-124 suppresses migration and invasion of GC cells. A, effects of miR-124 on migration of SGC-7901 cell by wound-healing; B, effects of miR-124 on invasion of SGC-7901 cell by transwell assay; C, statistical analysis of the results of wound-healing; D, statistical analysis of the results (B) of transwell assay; E, effects of miR-124 on migration of AGS cell by wound-healing; F, effects of miR-124 on invasion of AGS cell by transwell assay; G, statistical analysis of the results (E) of wound-healing; H, statistical analysis of the results (F) of transwell assay. Each group had three samples, and each sample was repeated three times. One-way ANOVA was used for statistical analysis; *, $P < 0.05$, compared with the control group; #, $P < 0.05$, compared with the mimic-NC group; Δ, $P < 0.05$, compared with the inhibitor-NC group; &, $P < 0.05$, compared with the miR-124 inhibitor group; miR-124, hsa-miR-124-3p; NC, negative control; GC, gastric cancer.
groups (all $P > 0.05$). When compared with the mimic-NC and control groups, the miR-124 mimic group exhibited higher cell apoptosis while the miR-124 inhibitor group exhibited a significant decrease in cell apoptosis. Cell apoptosis was higher in the miR-124 inhibitor + si-JAG1 group compared to the miR-124 inhibitor group (all $P < 0.05$). Expression of apoptosis-related proteins (Caspases-3, Bax, and Bcl-2) was detected using a Western blot assay. There were no evident differences in the expression of Caspases-3, Bax, and Bcl-2 among the control, mimic-NC, inhibitor-NC, and miR-124 inhibitor + si-JAG1 groups (all $P > 0.05$). In contrast to the control and mimic-NC groups, the miR-124 mimic group exhibited a higher expression of Caspases-3, Bax, and a lower expression of Bcl-2. As compared with the control and inhibitor-NC groups, the expression of Caspases-3 and Bax was significantly down-regulated and the expression of Bcl-2 was up-regulated in the miR-124 inhibitor group. In comparison with the miR-124 inhibitor group, the miR-124 inhibitor + si-JAG1 group exhibited an increased expression of Caspases-3 and Bax but a decreased expression of Bcl-2 (all $P < 0.05$) (Figure 8). The results suggested that miR-124 promoted the apoptosis of GC cells, increased the expression of Caspases-3 and Bax (pro-apoptotic protein), and decreased the expression of Bcl-2 (anti-apoptotic protein).

### 4 | DISCUSSION

miRNAs are inhibitors of malignant development of human cancer by means of targeting tumor suppressor genes or oncogenes. A previous study reported that miR-34a could target DLL1 gene, and up-regulation of miR-34a could influence cell proliferation and apoptosis by targeting DLL1. Therefore, we aimed to investigate the effects of miR-124 on GC through targeting JAG1 via the Notch signaling pathway.

We found that lower miR-124 expression and higher JAG1 expression in GC tissues were related to the clinicopathological features of GC patients. The tumor suppressor of miR-124 plays an essential role in the progression of GC. In line with our study, Jiang et al. demonstrated that the expression of miR-124 was negatively associated with JAG1 in GC tissues and adjacent tissues. Meanwhile, lower miR-124 expressions and higher JAG1 expressions in cancer tissues were significantly correlated with patients’ differentiation degree, lymph node metastasis, clinical stage, and infiltration degree. Additionally, our results found that up-regulation of miR-124 could inhibit the activity of the Notch signaling pathway. The NICD, an activated form of the Notch receptor, could be released by the activated Notch signaling pathway so that HES family expressions such as HES1 and HES5 could be increased. HES1 and HES5, essential to the Notch signaling pathway, could suppress cell apoptosis and promote invasion, migration, and proliferation. NICD, HES1, and HES5 were found to be down-regulated by miR-124 and activation of Notch signaling pathway, consistent with the results of our study. Gallardo et al. also demonstrated that up-regulated Notch related-RNA such as miR-124 could down-regulate the expression of JAG1 by suppressing the activity of the Notch signaling pathway.

Moreover, our study revealed that miR-124 could target JAG1 negatively, and higher expression of miR-124 could inhibit invasion, migration, and proliferation, and promotes apoptosis in human GC cells. MiR-124 could suppress GC cell proliferation and invasion, as well as induce GC cell apoptosis by inhibiting the EZH2 or SPHK1 directly, which could be a novel target for GC treatment. Consistently, miR-124 is proven to be a tumor suppressor by targeting ROCK1 so as to inhibit GC cell growth and invasion. A previous study reported that miR-124 was able to inhibit GC cell proliferation and promote cell apoptosis in way of targeting EZH2 and ROCK1 genes. Meanwhile, JAG1 could serve as a target of miR-598 or miR-34a or miR-21 in the colorectal cancer and monocyte-derived dendritic cell (MDDC) differentiation, indicating that JAG1 could be targeted by miRs. Patients suffering from cancer exhibiting a higher expression of JAG1 and Notch signaling pathway-related proteins had evidently poorer survivals. The inhibition of Notch signaling pathway

### TABLE 3 | Cell cycle distribution after transfection among six groups (%)

<table>
<thead>
<tr>
<th>Cell</th>
<th>Stage</th>
<th>The control group</th>
<th>The mimic-NC group</th>
<th>The miR-124 mimic group</th>
<th>The inhibitor-NC group</th>
<th>The miR-124 inhibitor + si-JAG1 group</th>
</tr>
</thead>
<tbody>
<tr>
<td>SGC-7901</td>
<td>Stage G0/G1</td>
<td>50.2 ± 0.8</td>
<td>50.8 ± 2.4</td>
<td>78.5 ± 1.1,*</td>
<td>49.2 ± 0.8</td>
<td>27.1 ± 0.3,*</td>
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<tr>
<td></td>
<td>Stage S</td>
<td>40.9 ± 0.6</td>
<td>40.8 ± 3.8</td>
<td>20.4 ± 0.5,*</td>
<td>42.5 ± 1.2</td>
<td>56.7 ± 0.9,*</td>
</tr>
<tr>
<td></td>
<td>Stage G2/M</td>
<td>8.9 ± 1.3</td>
<td>8.4 ± 1.5</td>
<td>1.1 ± 0.8,*</td>
<td>8.3 ± 1.8</td>
<td>16.2 ± 0.7,*</td>
</tr>
<tr>
<td>AGS</td>
<td>Stage G0/G1</td>
<td>54.2 ± 0.5</td>
<td>55.0 ± 1.0</td>
<td>81.5 ± 1.4,*</td>
<td>55.2 ± 0.5</td>
<td>32.1 ± 0.3,*</td>
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<tr>
<td></td>
<td>Stage S</td>
<td>37.9 ± 0.2</td>
<td>38.3 ± 1.2</td>
<td>15.6 ± 0.4,*</td>
<td>37.5 ± 1.5</td>
<td>50.4 ± 0.5,*</td>
</tr>
<tr>
<td></td>
<td>Stage G2/M</td>
<td>7.9 ± 0.5</td>
<td>6.7 ± 0.9</td>
<td>2.9 ± 1.1,*</td>
<td>7.3 ± 1.6</td>
<td>17.5 ± 0.8,*</td>
</tr>
</tbody>
</table>

Each sample was repeated three times; one-way ANOVA was used for statistical analysis. miR-124, hsa-miR-124-3; JAG1, jagged1; NC, negative control. *P < 0.05, compared with the control group; *P < 0.05, compared with the mimic-NC group; *P < 0.05, compared with the inhibitor-NC group; *P < 0.05, compared with the miR-124 inhibitor group.
miR-124 promotes apoptosis of GC cells. A, effects of miR-124 on apoptosis of SGC-7901 cell by flow cytometry; B, histogram of apoptosis of SGC-7901 cell; C, expression of apoptosis-related proteins of SGC-7901 cell detected by Western blotting assay; D, gray value analysis of nine Western blots of protein expression in panel C. E, effects of miR-124 on apoptosis of AGS cell by flow cytometry; F, histogram of apoptosis of AGS cell; G, expressions of apoptosis-related proteins of AGS cell detected by Western blotting assay; H, gray value analysis of nine Western blots of protein expression in panel G. Each group had three samples, and each sample was repeated three times. One-way ANOVA was used for statistical analysis; *, $P < 0.05$, compared with the control group; #, $P < 0.05$, compared with the mimic-NC group; Δ, $P < 0.05$, compared with the inhibitor-NC group; & $P < 0.05$, compared with the miR-124 inhibitor group; miR-124, hsa-miR-124-3p; NC, negative control; GC, gastric cancer.
activity may suppress GC cell invasion, migration, and proliferation, similar to our study.\textsuperscript{38} Also, as a terminal enzyme during cell apoptosis, caspase-3 could be downregulated by miR-124 mimics, and the intervention of miR-124 mimics could increase the expression of Bcl-2 but decrease the expression of Bax.\textsuperscript{39} Besides, Bcl-2 expression can directly prevent cancer cell apoptosis, and up-regulated Bcl-2 was seen in cancer cells when the activity of Notch signaling pathway was increased.\textsuperscript{40} Moreover, miR-421 could inhibit GC cell apoptosis by suppressing the expression of caspase-3.\textsuperscript{41}

Consequently, the present study found that miR-124 targets the JAG1 gene by suppressing the Notch signaling pathway, thus inhibiting invasion, migration, and proliferation of GC cells. However, we were unable to provide more specific miRNA-targeted ways for cancer diagnosis and therapy due to the unclear delivery and modification methods of oligonucleotides. Up-regulation of miR-124 is clinically helpful in suppressing the development of GC cells, and further research on monocular mechanisms of miR-124-based GC therapeutics are necessary.

ACKNOWLEDGMENTS

This work was supported by a grant from the National Natural Science Foundation of China (no. 81303135; no. 81573749) and the Basic Science Research in Shaanxi Province of China (no. 2015JQ8288). We acknowledge and appreciate our colleagues for their valuable efforts and comments on this paper.

CONFLICTS OF INTEREST

None.

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**How to cite this article:** Xiao H-J, Ji Q, Yang L, Li R-T, Zhang C, Hou J-M. In vivo and in vitro effects of microRNA-124 on human gastric cancer by targeting JAG1 through the Notch signaling pathway. *J Cell Biochem.* 2018;119:2520–2534. [https://doi.org/10.1002/jcb.26413](https://doi.org/10.1002/jcb.26413)