Pre-Clinical Research Report

NLRC5 promotes cell migration and invasion by activating the PI3K/AKT signaling pathway in endometrial cancer

Yijun Fan, Zhen Dong, Yuchuan Shi, Shiyong Sun, Bing Wei and Lei Zhan

Abstract
Objective: NOD-like receptor family caspase recruitment domain family domain-containing 5 (NLRC5) is involved in the development of cancer. Our objective was to explore the role of NLRC5 in the progression of endometrial cancer (EC).
Methods: The roles of NLRC5 in migration and invasion of AN3CA EC cells were examined by cell wound-healing assay, Transwell migration, and invasion analysis. Overexpression of NLRC5 was achieved with NLRC5 plasmid, and knockdown of NLRC5 was achieved using small interfering (si)RNA-NLRC5 in AN3CA cells. The expression of NLRC5 was detected by immunohistochemical, western blot, and quantitative real-time PCR. LY294002 was used to inhibit the phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway.
Results: NLRC5 was downregulated in EC tissue compared with normal endometrium. Overexpression of NLRC5 led to upregulation of cell migration and invasion in AN3CA cells and expression of matrix metallopeptidase (MMP)-9. Inhibition of NLRC5 restricted migration and invasion of AN3CA cells and expression of MMP9. Overexpression of NLRC5 promoted the activation of PI3K/AKT signaling pathway. Inhibiting PI3K/AKT signaling pathway by using LY294002 blocked the positive role of NLRC5 in migration and invasion of AN3CA cells and expression of MMP9.
Conclusions: These results demonstrate that NLRC5 promotes EC progression by activating the PI3K/AKT signaling pathway.

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Introduction

Endometrial cancer (EC) is the most common gynecological malignancy in the developed world. The latest data estimate 61,880 new cases and 12,160 deaths in the United States in 2019. The mortality rate of EC is also increasing, with an average 1.9% change annually from 2007 to 2016.1 Risk factors in the development of EC include polycystic ovarian syndrome, tamoxifen therapy, anovulation, nulliparity, early age of menarche, later age of menopause onset, Lynch syndrome, and obesity.2–4 EC is usually treated primarily with surgery; total hysterectomy and removal of both tubes and ovaries represent the standard approach in treating EC. Although hysterectomy results in excellent cancer-related outcomes for patients with early-stage EC, the prognosis for patients with advanced stage disease and high-risk histological subtypes remains poor.5 Thus, elucidating the molecular mechanism of EC is crucial for the prevention and treatment of the disease.

Pattern recognition receptors (PRRs) are critical sensors in the recognition of pathogen-associated molecular patterns (PAMPs) and play dual roles in the regulation of tumor immunity.6,7 For example, they can inhibit tumorigenesis by inducing immunogenic tumor cell death. They can also facilitate tumor growth through induction of the regulatory cell population and immunosuppressive cytokines.8,9 The NOD-like receptor (NLR) family, caspase activation and recruitment domain (CARD) domain-containing 5 (NLRC5) is a newly found member of the NLR family, which contains more than 20 members in the mammalian genome.10,11 Notably, recent studies have revealed controversial and alternating roles of NLRC5 in innate and adaptive immunity. For example, Cui et al. found that NLRC5 inhibited nuclear factor-kappa B (NF-κB)-dependent responses by interacting with IKKa and IKKb and blocking their phosphorylation. NLRC5-specific short interfering (si)RNA knockdown enhanced the activation of NF-κB and the inflammatory response in RAW 264.7 cells.12 However, in LX-2 cells, Xu and co-workers found that overexpression of NLRC5 upregulated interleukin (IL)-6 and IL-1β secretion, and knockdown of NLRC5 by transfecting siRNA decreased IL-6 and IL-1β secretion.13 Accumulating studies present evidence that NLRC5 plays an important role in immune evasion of cancers, that NLRC5 is a target for immune evasion in cancer, and that a high level of NLRC5 is correlated with higher survival.14,15 Furthermore, studies have shown that NLRC5 contributes to tumorigenesis by promoting cell proliferation, migration, and invasion in cancers related to a high inflammatory state.16,17 For example, overexpression of NLRC5 promotes the proliferation, migration, and invasion of hepatocellular carcinoma cells (HCC) by activating the downstream Wnt/β-catenin signaling pathway.17 In line with this, NLRC5 has been shown to promote HCC tumor proliferation by promoting the AKT/VEGF-A signaling pathway.18 Han and co-workers, however, suggested that knockdown of NLRC5 restricted renal ischemia-reperfusion injury by activating

Keywords
NLRC5, endometrial cancer, migration, invasion, matrix metallopeptidase 9, phosphatidylinositol 3-kinase/AKT signaling pathway

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the phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway in vitro, indicating conflicting results about the role of NLRC5 in the PI3K/AKT signaling pathway. Nevertheless, the role and mechanism of NLRC5 in EC have been unclear until now. Furthermore, the exact role of NLRC5 in PI3K/AKT signaling pathway in EC needs to be validated.

The aim of our study was to uncover the molecular mechanism by which NLRC5 plays a role in EC. We used clinical tissue samples from patients with EC and an EC cell line, AN3CA, to demonstrate the roles of NLRC5 in progression of EC.

**Materials and methods**

**Human tissue samples**

Endometrial tissue specimens from 20 patients with EC obtained at primary surgery and control endometrial tissue specimens from 20 patients undergoing hysterectomy for nonmalignant indications were collected and processed at the Department of Gynecology and Obstetrics, The Second Affiliated Hospital of Anhui Medical University from January 2018 to January 2019. None of the patients had received chemotherapy or radiotherapy before surgery. All patients gave written informed content. The study was approved by the institutional review board of Anhui Medical University. The demographic characteristics of EC patients and of women in the control group are summarized in Table 1. The two groups were matched for age. Samples were collected, quickly placed in liquid nitrogen, and stored at −80°C for western blot assay (five EC tissue specimens and five control tissue specimens) and real-time quantitative reverse transcription-PCR (RT-qPCR) assay (five EC tissue specimens).

| Variables                      | Patients without EC (n = 20) | Patients with EC (n = 20) | t     | p    |
|-------------------------------|-----------------------------|--------------------------|-------|------|
| Age, years                    | 51.70 ± 3.74                | 53.95 ± 4.15             | 1.801 | 0.080|
| Menopausal status             |                             |                          |       |      |
| Postmenopausal, no. (%)       | 16 (80.0)                   |                          |       |      |
| Premenopausal, no. (%)        | 4 (20.0)                    |                          |       |      |
| Histology                     |                             |                          |       |      |
| Endometrioid                  | 20 (100.0)                  |                          |       |      |
| FIGO stage (2009)             |                             |                          |       |      |
| I                             | 8 (40.0)                    |                          |       |      |
| II                            | 10 (50.0)                   |                          |       |      |
| III                           | 2 (10.0)                    |                          |       |      |
| Histological grade            |                             |                          |       |      |
| G1                            | 11 (55.0)                   |                          |       |      |
| G2                            | 6 (30.0)                    |                          |       |      |
| G3                            | 3 (15.0)                    |                          |       |      |
| Myometrial invasion           |                             |                          |       |      |
| ≤50%                          | 15 (75.0)                   |                          |       |      |
| >50%                          | 5 (25.0)                    |                          |       |      |
| Lymphatic node metastasis     |                             |                          |       |      |
| Positive                      | 2 (10.0)                    |                          |       |      |
| Negative                      | 18 (90.0)                   |                          |       |      |

EC, endometrial cancer; FIGO: Federation International of Gynecology and Obstetrics.
and five control tissue specimens). Ten EC tissue specimens and 10 control tissue specimens were prepared for formalin-fixed, paraffin-embedded tissue blocks for subsequent histopathological and immunohistochemical assay.

**Cell cultures**

The human EC cell line AN3CA was obtained from the Shanghai Institute of Cell Biology of the Chinese Academy of Sciences (Shanghai, China). AN3CA cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA), and 1% penicillin/streptomycin (Invitrogen) in the incubator at 37°C under 5% CO₂. The membranes were then incubated in Tris-buffered saline-Tween (TBST; Boster Bio, Wuhan, China) containing 5% skim milk at 37°C for 4 hours, and with specific primary antibodies at 4°C overnight. The next day, the membranes were washed three times with TBS-Tween 20 (0.075%), followed by incubation with horseradish peroxidase-conjugated secondary antibodies (1:10,000) at 37°C for 1 hour. After washing three times with TBST, proteins were visualized using an ECL chemiluminescent kit (ECL-plus, Thermo Fisher Scientific, Waltham, MA, USA). All experiments were performed in triplicate and repeated at least three times.

**Protein extraction and western blot analysis**

Endometrial tissue specimens or AN3CA cells were lysed in protein extraction solution (Beyotime, Jiangsu, China). Lysates collected by centrifugation were applied on polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corp., Billerica, MA, USA). After blocking nonspecific protein binding, nitrocellulose blots were incubated for 6 hours with primary antibodies diluted in primary antibody dilution buffer (Beyotime). The primary antibodies recognizing NLRC5 (ab105411, Abcam, Cambridge, MA, USA), MMP9, and ACTB were used at 1:1,000, 1:1,000, and 1:3,000, respectively. Relative expression levels were calculated based on the standard 2⁻ΔΔCt method. All experiments were performed in triplicate and repeated at least three times. The RT-qPCR primer sequences used are as follows: NLRC5-forward: 5'-GGTTCTTAGGGTTC CGTCAGCG-3', NLRC5-reverse: 5'-CA GTCCTTCAGAGTGGCACAGAG-3'; MMP9-forward: 5'-ACGCACGACGTCT TCCAGTA-3', MMP9-reverse: 5'-CCAC CTGGTTCAACTCACTCC-3'; ACTB-forward: 5'-CACCAGCACAATGAAG ATCAAGAT-3', ACTB-reverse: 5'-CCAGTTTTTAAATCCTGAGTCAAGC-3'.

**RNA isolation and RT-qPCR**

Total RNA was extracted using TRIzol total RNA isolation reagents (Invitrogen), and first-strand cDNA was synthesized from total RNA using a Thermoscript RT-PCR synthesis kit (Fermentas, Burlington, ON, Canada) in accordance with the manufacturer’s instructions. RT-qPCR analyses for mRNA of NLRC5, MMP9, and ACTB were carried out by using Thermoscript RT-qPCR kits in an ABI Prism Step-One Plus real time PCR System (Applied Biosystems, Foster City, CA, USA). The mRNA level of ACTB was used as an internal control. Relative expression levels were calculated based on the standard 2⁻ΔΔCt method. All experiments were performed in triplicate and repeated at least three times. The RT-qPCR primer sequences used are as follows: NLRC5-forward: 5'-GGTTCTTAGGGTTC CGTCAGCG-3', NLRC5-reverse: 5'-CA GTCCTTCAGAGTGGCACAGAG-3'; MMP9-forward: 5'-ACGCACGACGTCT TCCAGTA-3', MMP9-reverse: 5'-CCAC CTGGTTCAACTCACTCC-3'; ACTB-forward: 5'-CACCAGCACAATGAAG ATCAAGAT-3', ACTB-reverse: 5'-CCAGTTTTTAAATCCTGAGTCAAGC-3'.
**Transient transfection of AN3CA cells of NLRC5 plasmid and siRNA-NLRC5**

The full-length coding region (1,276 bp) of NLRC5 was amplified from human genomic DNA by reverse transcription (RT)-PCR using the following primers: forward: 5'-CCGGAATTCGGATGGCAAGGAAGCTGGA-3' and reverse: 5'-GGGATCCCGTCACCTGAAGCTTCTTCCCA-3'.

The PCR products were digested with EcoRI/BamHI and were inserted into the pEGFP-C2 empty vector (Clontech, Shanghai, China). The recombinant construct pEGFP-C2-NLRC5 was verified by direct DNA sequencing. Cell transfection was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Small interfering (si) RNA oligonucleotides against NLRC5 or scrambled sequences were designed and synthesized by the Gema Pharma Corporation (Shanghai, China) and contained the following sequences: siRNA-NLRC5-sense: AAGAACGAGAGACUGCACACUGCdTdT, siRNA-NLRC5-antisense: GCAGUUGGCAGAGUCUCGUUdTdT, scrambled-RNAi-sense: UUCUCCGAACGUGACGUUGCACGU TT, and scrambled-RNAi-antisense: ACGUGACACGUUCCGAGAATT.

The AN3CA cells were cultured in 6-well plates with antibiotics-free DMEM for 24 hours and then transfected with siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol.

**Cell wound healing assay**

A wound healing assay was used to assess cell migration. After the appropriate treatments, cells (1 × 10^5) were trypsinized, seeded into a 6-well plate, and allowed to grow to 75% confluence in complete medium. Then, the cell layer was wounded using a sterile pipette tip, washed with PBS several times to remove cell debris, and incubated for 48 hours in serum-free medium. During incubation at 37°C, cells migrated into the wound surface, a process of in vitro healing. The wound healing in vitro was photographed by using an inverted fluorescence microscope and the rate of closure was assessed, as follows.

Rate of wound healing = [(wound width at 0 hours – wound width at 48 hours)/0-hour wound width] × 100%.

**Cell Transwell migration and invasion analysis**

A 24-well Transwell Boyden chamber (Corning Inc., Corning, NY, USA) with an 8.0-μm pore size polycarbonate membrane was used for the migration and invasion assay, according to the manufacturer’s protocol. For the migration assay, after appropriate treatments, cells were trypsinized and seeded in the upper chamber at a density of 1 × 10^5 cells/well in 100 μL of serum-free medium. Then, 600 μL of complete medium was added to the lower chamber as a chemoattractant. After incubation for 24 hours at 37°C, cells remaining at the upper surface of the membrane were removed with cotton swabs. The cells on the lower surface of the membrane represent the migrated cells. After fixation with 4% paraformaldehyde and staining with crystal violet solution, cells that passed through the filter were photographed by using an inverted fluorescence microscope. The cell invasion assay was carried out similarly, except that 100 μL of 1:8 DMEM-diluted Matrigel (BD, Franklin Lakes, NJ, USA) was added to each well at 37°C for 6 hours before cells were seeded onto the membrane, followed by incubation for 48 hours.

**Treatment of AN3CA cells with PI3K/akt inhibitor LY294002**

The PI3K/akt inhibitor LY294002 (Sigma Chemical Co., St. Louis, MO,
USA) was dissolved in dimethyl sulfoxide (Sigma Chemical Co.). A previous study showed that 20 μM LY294002 could inhibit the PI3K/AKT pathway in EC cell lines. Furthermore, 10, 20, 30, and 40 μM LY294002 could inhibit survival of cancer cells significantly, including EC cell lines. Therefore, we used LY294002 at a concentration of 25 μM to inhibit the PI3K/AKT pathway in AN3CA cells. The AN3CA cells were seeded overnight in culture dishes and transfected with NLRC5 plasmid 6 hours later; then, AN3CA cells were treated with LY294002 for 48 hours.

**Statistical analysis**

All data were analyzed by the SPSS version 17.0 software (SPSS Inc., Chicago, IL, USA) and presented as means ± standard deviations. The statistical significance of differences was determined by Student’s *t*-test for comparison between means or one-way analysis of variance (ANOVA). Data were regarded as statistically significant at *p* < 0.05 and **p** < 0.01.

**Results**

NLRC5 was downregulated in human EC endometrial tissues

An immunohistochemical assay was first performed to analyze the NLRC5 expression level in EC tumor and normal endometrium tissues. In Figure 1A, NLRC5 expression in EC tumor tissues was

![Figure 1](image-url)

**Figure 1.** NLRC5 was downregulated in human EC tissues. (A) The endometrial tissues of control patients and patients with EC were stained by immunohistochemistry with NLRC5 (100×). (B) The protein expression of NLRC5 in control endometrium tissues and EC tumor tissues; **p** < 0.01 vs control patients. (C) The mRNA expression of NLRC5 in control endometrium tissues and EC tumor tissues; **p** < 0.01 vs control patients. NLRC5, NOD-like receptor family caspase recruitment domain family domain-containing 5; EC, endometrial cancer.
significantly lower than the endometrium tissues of control patients \( (p < 0.01) \). Consistently, western blot (Figure 1B) and RT-qPCR (Figure 1C) analysis showed that the level of NLRC5 in EC tumor tissues was significantly downregulated compared with that in control tissues \( (p < 0.01) \). These results demonstrate that NLRC5 is involved in the progression of EC.

**Overexpression of NLRC5 enhanced cell migration and invasion of AN3CA cells**

To investigate the role of NLRC5 in EC, NLRC5 plasmid was constructed, and transfection was performed to overexpress NLRC5 protein and mRNA in AN3CA cells (Figure 2A and B, \( p < 0.01 \)). After transfection, wound healing and Transwell assays were used to assess cell migration and invasion. As shown in Figure 2C and D, overexpression of NLRC5 enhanced migration and invasion of AN3CA cells \( (p < 0.01) \). Furthermore, as shown in Figure 2E and F, overexpression of NLRC5 enhanced expression of MMP9 in AN3CA cells \( (p < 0.01) \).

**Inhibition of NLRC5 repressed cell migration and invasion of AN3CA cells**

To further investigate the role of NLRC5 in EC, NLRC5 siRNA was constructed, and transfection was performed to inhibit NLRC5 protein and mRNA in AN3CA cells (Figure 3A and B, \( p < 0.01 \)). After transfection, wound healing and Transwell assays were used to detect cell migration and invasion. As shown in Figure 3C and D, inhibition of NLRC5 repressed migration and invasion of AN3CA cells \( (p < 0.05 \) and \( p < 0.01 \), respectively). Furthermore, as shown in Figure 3E and F, inhibition of NLRC5 restricted the expression of MMP9 in AN3CA cells \( (p < 0.01) \).

**NLRC5 promoted AN3CA cell migration and invasion by activating the PI3K/AKT signaling pathway**

To investigate the molecular mechanism of NLRC5 in EC, we first found that overexpression of NLRC5 led to activation of the PI3K/AKT signaling pathway (Figure 4A, \( p < 0.01 \)), and downregulation of NLRC5 inhibited the PI3K/AKT signaling pathway (Figure 4B, \( p < 0.01 \)). Furthermore, the PI3K/AKT signaling pathway inhibitor LY294002 restricted the role of NLRC5 in the PI3K/AKT signaling pathway (Figure 4C, \( p < 0.01 \)). In addition, LY294002 blocked cell migration and invasion in AN3CA cells overexpressing NLRC5 (Figure 4D and E, \( p < 0.01 \)). Consistently, LY294002 inhibited the expression of MMP9 in AN3CA cells overexpressing NLRC5 (Figure 4F and G, \( p < 0.01 \)). These results demonstrate that NLRC5 promotes EC cell migration and invasion by activating the PI3K/AKT signaling pathway.

**Discussion**

In the present study, we demonstrated that NLRC5 was downregulated in endometrium of EC patients. Overexpression of NLRC5 promoted migration and invasion of AN3CA cells, and downregulation of NLRC5 inhibited migration and invasion of AN3CA cells. Furthermore, we found that NLRC5 promotes migration and invasion of AN3CA cells by activating the PI3K/AKT signaling pathway.

PRRs are critical sensors in PAMP recognition, which provide the first line of defense against invading microbes in the innate immune response.\(^{22,23}\) The NOD-like receptors are a recently discovered family of cytoplasmic PRRs. NLRs are evolutionarily conserved proteins that sponsor the induction of innate and adaptive immunity.\(^{24,25}\) Recently, it has been shown that NLRs play roles in cancer and
Figure 2. Overexpression of NLRC5 enhanced cell migration and invasion of AN3CA cells. (A, B) AN3CA cells were transfected with NLRC5 plasmid and NLRC5 protein and mRNA expression was measured in AN3CA cells; **p < 0.01 vs. vector group. (C) Wound healing assay indicated that migration of AN3CA cells was upregulated after transfection with NLRC5 plasmid; **p < 0.01 vs. vector group. (D) Transwell invasion assays showed that overexpression of NLRC5 increased the invasion rate in AN3CA cells; **p < 0.01 vs. vector group. (E, F) MMP9 protein and mRNA expression was measured in AN3CA cells; **p < 0.01 vs. vector group. NLRC5, NOD-like receptor family caspase recruitment domain family domain-containing 5; AN3CA, endometrial cancer cell line; MMP9, metallopeptidase 9.
Figure 3. Inhibition of NLRC5 repressed cell migration and invasion of AN3CA cells. (A, B) AN3CA cells were transfected with NLRC5-siRNA, NLRC5 protein and mRNA expression was measured in AN3CA cells; $**p < 0.01$ vs. scrambled siRNA group. (C) Wound healing assay indicated that migration of AN3CA cells was downregulated after transfection with NLRC5-siRNA; $*p < 0.05$ vs. scrambled siRNA group. (D) Transwell invasion assays showed that inhibition of NLRC5 repressed invasion rate in AN3CA cells; $**p < 0.01$ vs. scrambled siRNA group. (E, F) MMP9 protein and mRNA expression was measured in AN3CA cells; $**p < 0.01$ vs. scrambled siRNA group. NLRC5, NOD-like receptor family caspase recruitment domain family domain-containing 5; AN3CA, endometrial cancer cell line; NLRC-siRNA, NLRC5 short interfering RNA; MMP9, metallopeptidase 9.
Figure 4. NLRC5 promoted AN3CA cell migration and invasion by activating PI3K/AKT signaling pathway. (A) AN3CA cells were transfected with NLRC5 plasmid and phosphorylated (p)-PI3K and p-AKT protein expression was measured in AN3CA cells; **p < 0.01 vs. vector group. (B) AN3CA cells were transfected with NLRC5-siRNA and p-PI3K and p-AKT protein expression was measured in AN3CA cells; **p < 0.01 vs. scrambled siRNA group. (C) NLRC5 plasmid-transfected AN3CA cells were treated with 25 μM LY294002 for 48 hours. Protein expression of p-PI3K and p-AKT was assessed by western blot; **p < 0.01 vs. NLRC5 plasmid group. (D) NLRC5 plasmid-transfected AN3CA cells were treated with 25 μM LY294002 for 48 hours. Wound healing assay indicated that migration of NLRC5 plasmid-transfected AN3CA cells was downregulated after treatment with 25 μM LY294002; **p < 0.01 vs. vector group, ##p < 0.01 vs. NLRC5 plasmid group. (E) NLRC5 plasmid-transfected AN3CA cells were treated with 25 μM LY294002 for 48 hours. Transwell invasion assays showed that inhibition of PI3K/AKT signaling pathway repressed the invasion rate in NLRC5 plasmid-transfected AN3CA cells; **p < 0.01 vs. vector group, ##p < 0.01 vs. NLRC5 plasmid group. (F, G) Protein expression of MMP9 was assessed by western blot and RT-qPCR; **p < 0.01 vs. vector group, ##p < 0.01 vs. NLRC5 plasmid group. NLRC5, NOD-like receptor family caspase recruitment domain family domain-containing 5; AN3CA, endometrial cancer cell line; NLRC-siRNA, NLRC5 short interfering RNA; MMP9, metallopeptidase 9; LY294002, inhibitor of PI3K/AKT signaling pathway.
inflammation. NLRC5 is a novel member of the NLR family; it possesses three structural domains: the N-terminal atypical CARD, which is completely distinct from the other NLRs; the central NACHT domain, which contains the nucleotide binding domain; and 27 leucine-rich repeats at the C-terminal. NLRC5 has the longest leucine-rich repeat domain of all human NLR proteins and is the largest member of the NLR family, with a predicted size of more than 200 kDa. NLRC5 is a transcriptional coactivator of the major histocompatibility complex (MHC) class I antigen presentation pathway, suggesting a critical role of NLRC5 in the human adaptive immune system. Accumulating evidence confirms that NLRC5 has a close relationship with cancer. On the one hand, NLRC5 is downregulated and acts as a target for immune evasion in cancer, and high levels of NLRC5 are correlated with higher survival of cancer patients. On the other hand, novel studies have shown that NLRC5 is upregulated and implicated in tumorigenesis by promoting cell proliferation, migration, and invasion in high inflammatory and immune state–related cancers. However, the role and mechanism of NLRC5 in EC is unclear. In our study, we found that NLRC5 was downregulated in endometrium of EC patients compared with that of patients without EC. Furthermore, overexpression of NLRC5 promoted migration and invasion of AN3CA cells and expression of MMP9. Downregulation of NLRC5 inhibited migration and invasion of AN3CA cells and expression of MMP9. These results are consistent with the role of NLRC5 in other cancers, including HCC and renal cancer.

Activation of the PI3K/AKT signaling pathway is considered a marker of cancer progression, including that in EC. Previous studies have suggested a close relationship between NLRC5 and the PI3K/AKT signaling pathway. However, NLRC5 has been reported to both promote and inhibit PI3K/AKT signaling in different diseases. Here, our data suggested that NLRC5 can promote migration and invasion of AN3CA cells by stimulating the PI3K/AKT signaling pathway. Furthermore, when the PI3K/AKT signaling pathway was inhibited by its specific inhibitor LY294002, the role of NLRC5 in migration and invasion of AN3CA cells was abolished.

The aim of this study was to investigate whether NLRC5 promoted cell migration and invasion of EC cells by targeting the PI3K/AKT signaling pathway in vitro. Further research should examine the mechanism of NLRC5 in the regulation of cell migration and invasion in other EC cell lines. Moreover, these effects should be investigated and confirmed in vivo. We found that NLRC5 was downregulated in EC but played a positive role in EC progression. We propose that DNA methylation at a CpG island in the NLRC5, copy number loss of the NLRC5 gene, or somatic mutations in NLRC5 may lead to NLRC5 downregulation in EC compared with normal tissue, although this hypothesis requires further validation.

In summary, we demonstrated that expression of NLRC5 was low in endometrial tissue of patients with EC. Overexpression of NLRC5 promoted migration and invasion of AN3CA cells and expression of MMP9, whereas downregulation of NLRC5 inhibited migration and invasion of AN3CA cells and expression of MMP9. The PI3K/AKT signaling pathway is involved in the role of NLRC5 in migration and invasion. These findings indicate that NLRC5 might be a novel therapeutic target for the treatment of EC.

Authors’ contributions
YJF and LZ wrote the manuscript, YCS and SYS performed the statistical analysis, and WB and LZ supervised all protocols. All authors...
read, provided feedback, and approved the final manuscript.

**Declaration of conflicting interest**
The authors declare that there is no conflict of interest.

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