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ORIGINAL ARTICLE

miR-34a-5p Inhibits Cell Proliferation, Migration and Invasion Through Targeting JAG1/Notch1 Pathway in HPV-Infected Human Epidermal Keratinocytes

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Abstract

Condyloma acuminate (CA) is a communicable disease caused by human papillomavirus (HPV). This study aimed to study the targeting relationship between miR-34a-5p and Jagged 1 (JAG1), as well as its regulatory effect in HPV-infected cells. Human keratinocyte HaCaT cells were infected with HPV16E6, and CA tissues were collected. The expression level of miR-34a-5p and JAG1 were detected in CA tissues and HPV-HaCaT cells. Cell proliferation, migration and invasion were respectively measured using 3-(4, 5)-dimethylthiahiazo-(−z-y1)-3, 5-diphenytetrazoliumromide (MTT), cell wound healing and Transwell assay. The potential binding sites of miR-34a-5p and JAG1 were predicted by website TargetScan, and confirmed using dual luciferase reporter gene assay. The proteins of Notch1 pathway-related were assessed using western blotting. The results showed that miR-34a-5p expression was decreased, and JAG1 expression was increased in CA tissues and HPV-HaCaT cells. Cell proliferation, migration and invasion were decreased when miR-34a-5p over-expression and JAG1 knock-down in HPV-HaCaT cells. Furthermore, miR-34a-5p had a targeting effect on JAG1. The expression level of Notch1, NICD, Hes1 and Hey1 were increased when miR-34a-5p knock-down. miR-34a-5p could inhibit cell development, and regulate the activity of Notch1 pathway through targeting JAG1 expression in HPV-infected keratinocytes.

Keywords miR-34a-5p · Jagged 1 (JAG1) · Notch1 pathway · Human papillomavirus (HPV) · Condyloma acuminate (CA)

Introduction

Condyloma acuminate (CA) is a communicable disease of the perianal genital area caused by human papillomavirus (HPV) infection of the skin and mucosa, which is characterized by rapid growth, high infectivity and easy recurrence in the early stage of treatment [1, 2]. Previous studies on the pathogenesis of CA have focused on abnormal proliferation, apoptosis, cellular immunity and changes in humoral immune function [3, 4]. However, the development of CA is a complicated process of multi-factor and multi-gene regulation [5]. Therefore, it is a hot spot for researchers to find possible regulatory targets of CA [2, 5].

microRNAs (miRNAs) are a class of single-stranded non-coding RNAs with the length of 20–25 nt, which have stability and specificity [6]. In recent years, it has been suggested that miRNAs are closely related to viral infection and are involved in the pathogenesis of the virus [7, 8]. Virus-induced changes in host miRNAs expression and virus-encoded miRNAs could regulate differentiation, proliferation and apoptosis of host cells, which is one of the mechanisms of viral pathogenesis. Furthermore, HPV infection is connected with the occurrence of malignant tumors, such as cervical cancer, oral squamous cell carcinoma and neck cancer [9–11], and can cause the changes of miRNA expression profiles in cancer cells [11, 12]. Mckenna et al. and Marthaler AM et al. showed that miR-24, miR-205 and miR-203 expressions were affected by HPV in human keratinocytes [13, 14]. Moreover, it has been reported that HPVE6 or HPVE7 regulate miR-23b, miR-34a or miR-15a/miR-16-1 expressions

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through blocking the expression of intracellular transcription factors, like p53 and pRB [15–17]. Among them, miR-34a-5p is a anti-oncogene in various tumors and can inhibit cancer cell development [18]. However, the effect of miR-34a-5p on the pathogenesis of CA is unclear.

miRNAs are not an independent regulatory factor, which can regulate genes at transcriptional level by complementary pairing with target gene mRNA, causing the inhibition of mRNA translational or degradation [19]. Pu et al. reported that miR-34a-5p promotes chemotherapeutic resistance of osteosarcoma cells via regulating AGTR1 gene expression [18]. Moreover, Jagged 1 (JAG1) has also been reported to be a target gene of miR-34a, but the regulatory function of miR-34a-5p in HPV-induced CA targeting JAG1 have not been reported [20].

Therefore, in this study, we aimed to explore the targeting relationship and regulatory effect of miR-34a-5p on JAG1 in vitro, and to clarify the value of miR-34a-5p in the treatment of CA induced by HPV. The results showed that miR-34a-5p inhibited cell development, and regulate the activity of Notch1 pathway through targeting JAG1 expression in HPV-infected keratinocytes. This study will provide a new target for effective CA treatment.

Materials and Methods

Tissues Collection and Cell Culture

CA tissues were taken from the vulva and anus of 36 patients (23 male and 13 female), who were diagnosed by histopathology between October 2017 and July 2018 in The Central hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology. The patient's course of this disease was 1–6 months, with an average of 3.1 months. Normal skin (NS) tissues were obtained from the normal foreskin of patients ($n = 15$) with circumcision in the outpatient operating room of the same hospital. The patients were informed and agreed with tissue extraction. All experiment protocols were approved by the Ethics Committee of The Central hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology.

Human keratinocyte cell line HaCaT (Cat No.: 300493) was purchased from Cell Lines Service (CLS, Eppelheim, Germany) and cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, California, USA) containing 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, USA) at 37 °C.

Transformed cell line 293 T (Cat No.: CL-0005) was purchased from Procell Life Science & Technology Co., Ltd. (Wuhan, China) and cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin (P/S, Thermo Fisher Scientific, Waltham, USA).

Construction of HPV16E6 Keratinocyte Model

HPV16E6 gene was artificially synthesized and pEGFP-N1-HPV16E6 recombinant plasmid vector was constructed by Sangon Biotech Co., Ltd. (Shanghai, China). The recombinant plasmids were transformed into competent cells, and then transformed cells were incubated at 37 °C for 12–16 h. The recombinant plasmids were digested by Hind III and Xba I, and the DNA sequence was identified by Sangon Biotech Co., Ltd. Based on the specification, pEGFP-N1-HPV16E6 plasmid was transfected into HaCaT cells using Lipofectamine 3000 Reagent (Thermo Fisher Scientific, Waltham, USA) to construct the HPV16E6 keratinocyte model (HPV-HaCaT).

Cell Transfection

Cells (2×10^5 cells/well) were seeded into 6-well plates for 24 h at 37 °C. miR-34a-5p mimics, miR-34a-5p-inhibitor, NC-mimics or NC-inhibitor were transfected into HaCaT cells using Lipofectamine 3000 Reagent (Thermo Fisher Scientific, Waltham, USA). Similarly, small interfering RNA (siRNA) JAG1 (JAG1-siRNA), JAG1 or NC-siRNA were transfected into HaCaT cells, which represented JAG1 knock-down or over-expression using Lipofectamine 3000 Reagent (Thermo Fisher Scientific, Waltham, USA) according to the instruction.

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from HaCaT cells using Invitrogen Trizol Reagent (Invitrogen Life Technologies, Carlsbad, USA), and then cDNA was synthesized using SuperScript™ VILO™ cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, USA). The fragments of miR-34a-5p and JAG1 were obtained by PCR amplification. The PCR reaction conditions were as follows: 95 °C for 10 min; 35 cycles of 95 °C for 30 s, 60 °C for 10s and 72 °C for 10s; finally 72 °C for 10 min. The results were analyzed by $2^{-\Delta\Delta Ct}$ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal control [21].

Western Blotting

Total protein was extracted from HaCaT cells and quantitatively detected using BCA BCA Protein Assay Kit (Beyotime, Shanghai, China). The proteins were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to the Polyvinylidene Difluoride (PVDF) membranes. After washing the membranes with phosphate buffer saline (PBS), the proteins were sealed with 5% skim milk for 1 h and added with antibodies (1:1000; monoclonal rabbit antibody; JAG1, Notch1, NICD, Hes1 and Hey; Abcam, Shanghai, China) at 4 °C overnight.

Secondary anti-rabbit IgG antibody (1:2000; Abcam, Shanghai, China) marked by horseradish peroxidase (HRP) was added into the membranes, and incubated for 30 min at normal temperature. After washing membranes, the proteins were detected by BeyoECL Plus Kit (Beyotime, Shanghai, China). The optical density was analyzed by Quantity One software, while β-actin was used as internal reference.

Cell Proliferation Assay

Cell proliferation assay was assessed by 3-(4, 5)-dimethylthiahiazo-(−z-y1)-3, 5-diphenytetrazoliumromide (MTT) assay. Cells (5×10^3 cells/well) were firstly seeded into 96-well plates for 48 h at 37 °C, and sequentially incubated in DMEM with 0.5 mg/mL MTT solution for 4 h. After adding dimethylsulfoxide (DMSO), the samples were measured at the wavelength of 490 nm (OD490) using Enzyme-linked immunodetector (Thermo Fisher Scientific, Waltham, USA).

Cell Migration Assay

Cell migration assay was assessed by cell wound healing assay. Cells (2×10^5 cells/well) were seeded into 6-well plates with 5 mm horizontal line for 24 h at 37 °C. After washing in PBS, cells were incubated on vertical horizontal plates for 24 h at 37 °C. The relative Gap width was measured and recorded in 0 h and 24 h using a Nikon Microscope (Nikon, Tokyo, Japan).

Cell Invasion Assay

Cell invasion assay was assessed by Transwell assay. Cells (5×10^4 cells/well) were seeded into 24-well plates at 37 °C for 24 h. The upper chamber of the bottom membrane of the Transwell chamber was coated with Matrigel (1:8; BD biosciences, California, USA) for 1–4 h at 37 °C. The cell suspension were added into Transwell chamber, and incubated for 24 h at 37 °C. Washed cells were fixed with methanol for 30 min and stained with 0.1% crystal violet (Thermo Fisher Scientific, Waltham, USA) for 30–60 min. The traversed cells were counted in five visual fields under the 400× microscope (Leica Microsystems, Weitzlar, Germany).

Dual Luciferase Reporter Gene Assay

The binding sites of miR-34a-5p and JAG1 were predicted by website TargetScan (http://www.targetscan.org). Then, miR-34a-5p mimics, miR-34a-5p inhibitor, NC-mimics or NC-inhibitor and JAG1 3'UTR-wt or JAG1 3'UTR-mutation (mut), which were designed by GenePharma Co., Ltd. (Shanghai, China), were co-transfected into 293 T cells using Lipofectamine 3000 Reagent (Thermo Fisher Scientific, Waltham, USA). The dual luciferase activities were measured using Dual-Luciferase® Reporter Assay System Protocol (Promega, Madison, USA).

Statistical Analysis

The data were analyzed by statistical software SPSS 19.0 (SPSS Inc., Chicago, USA) and showed using the mean value ± standard deviation (SD). The student's t test was used for inter-group comparison. $p < 0.05$ was considered as statistically significant (*$p < 0.05$, **$p < 0.01$ or ***$p < 0.001$).

Results

miR-34a-5p and JAG1 Were Abnormally Expressed in CA Tissues and HPV-HaCaT Cells

In the study, we constructed HPV16E6 plasmid to transfect HaCaT cells and got CA tissues in order to detect miR-34a-5p and JAG1 expression using qRT-PCR and western blotting. The results displayed that miR-34a-5p mRNA expression level in CA group was lower than that in NS group ($p < 0.001$, Fig. 1a). JAG1 protein expression level was up-regulated in CA tissues ($p < 0.001$, Fig. 1b). Subsequently, as shown in Fig. 1c, miR-34a-5p mRNA expression level was decreased when HPV16E6 transfected HaCaT cells ($p < 0.001$). Conversely, JAG1 expression was obviously up-regulated in HPV-HaCaT group compared with HaCaT group ($p < 0.001$, Fig. 1d).

miR-34a-5p Inhibited Cell Development in HPV-HaCaT Cells

To assess the effect of miR-34a-5p on cell growth, miR-34a-5p mimics or miR-34a-5p inhibitors were transfected into HPV-HaCaT cells. In Fig. 2a, the data suggested that miR-34a-5p mimics and inhibitors were transfected into the HPV-HaCaT cells efficiently and successfully ($p < 0.001$). MTT assay results showed that cell proliferation was decreased when miR-34a-5p over-expression, while was increased when miR-34a-5p knock-down in HPV-HaCaT cells ($p < 0.001$, Fig. 2b). Cell wound healing assay results displayed that the relative gap width in miR-34a-5p mimics group was wider than that in NC mimics group after incubation for 24 h, but gap width was significantly reduced when knocked down miR-34a-5p (Fig. 2c). Moreover, Transwell assay results revealed that invasion cell number was clearly reduced in HPV-HaCaT cells were transfected with miR-34a-5p mimics ($p < 0.01$), conversely, it was increased in miR-34a-5p inhibitor group than NC inhibitor group ($p < 0.001$, Fig. 2d).

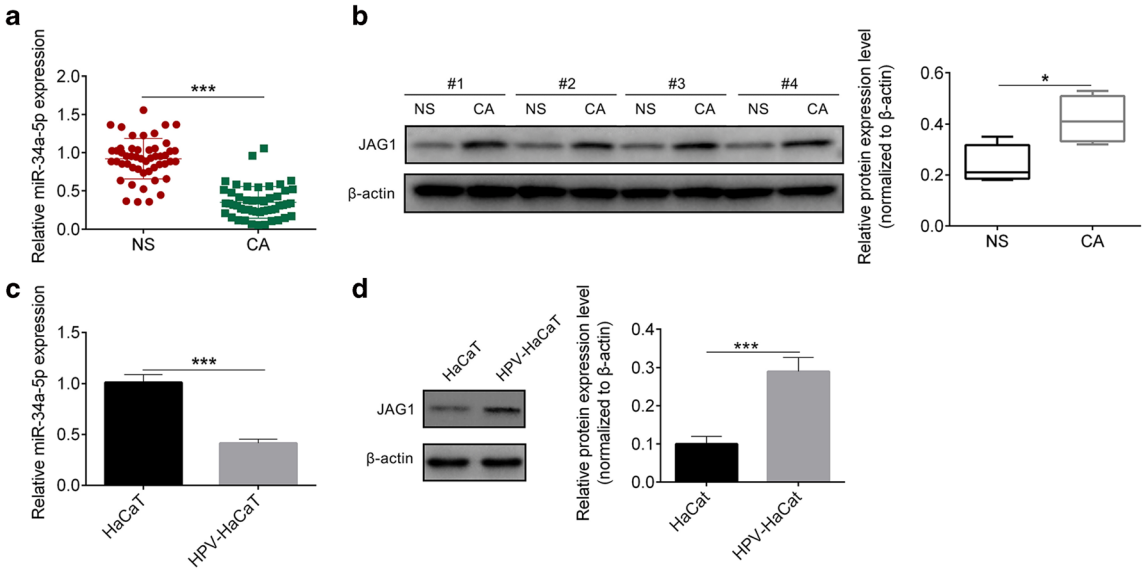

Fig. 1 miR-34a-5p and JAG1 were abnormally expressed in condyloma acuminate (CA) tissues and HPV-HaCaT cells. a The expression of miR-34a-5p was detected using qRT-PCR in CA tissue and normal skin (NS) tissue. **b** JAG1 expression was detected using western blotting in CA tissue and NS tissue. **c** The expression of miR-34a-5p was detected using qRT-PCR in HPV-HaCaT cells and HaCaT cells. **d** JAG1 expression was detected using western blotting in HPV-HaCaT cells and HaCaT cells. ***$p < 0.001$

JAG1 Promoted Cell Development in HPV-HaCaT Cells

Subsequently, in order to detect the effect of JAG1 on cell growth, JAG1 siRNA was transfected into HPV-HaCaT cells as JAG1 silencing. Figure 3a told that mRNA and protein expression levels were significantly decreased in JAG1 siRNA group, suggesting the transfection of JAG1 siRNA was successful ($p < 0.001$). Figure 3b showed that cell proliferation was reduced when JAG1 silencing ($p < 0.001$). Then, JAG1 silencing remarkably increased the relative gap with (Fig. 3c). Transwell assay results displayed that the invasion cell number in JAG1 siRNA group was less than that in NC siRNA group ($p < 0.001$, Fig. 3d). Sum up, JAG1 silencing inhibited HPV-HaCaT cell proliferation, migration and invasion.

miR-34a-5p Had a Targeting Effect on JAG1

In the study, we predicted the potential binding sequence of miR-34a-5p and JAG1 by bioinformatic analysis, and found that there is binding site between position 1302–1308 of JAG1 3′ untranslated regions (UTR) and has-miR-34a-5p (Fig. 4a). Subsequently, dual luciferase reporter gene assay results showed that co-transfection of miR-34a-5p mimics and JAG1 3'UTR-wt reduced the luciferase activity clearly, on the contrary, the luciferase activity was added in miR-34a-5p inhibitor and JAG1 3'UTR-wt co-transfected group than JAG1 3'UTR-wt and NC inhibitor co-transfected group ($p < 0.001$). However, co-transfection of JAG1 3'UTR-mut and miR-34a-5p mimics or JAG1 3'UTR-mut and miR-34a-5p inhibitor did not change any activity (Fig. 4b). In Fig. 4c, JAG1 protein expression level was down-regulated when miR-34a-5p over-expression, but was significantly up-regulated when miR-34a-5p knock-down ($p < 0.01$ or $p < 0.001$). These data indicated that miR-34a-5p could target JAG1 expression.

miR-34a-5p Inhibited Cell Development through Inhibiting JAG1 Expression

After miR-34a-5p and JAG1 were transfected into HPV-HaCaT cells, cell proliferation, migration and invasion were detected. From Fig. 5a, the results showed that JAG1 over-expression alleviated the inhibition of miR-34a-5p on cell proliferation ($p < 0.01$). Then, relative gap width in miR-34a-5p + JAG1 group was clearly more narrow than that in miR-34a-5p + control group, suggesting that cell migration inhibited by miR-34a-5p overexpression was abolished when JAG1 over-expression ($p < 0.001$, Fig. 5b). Similarly, Transwell assay also showed that miR-34a-5p and JAG1 co-over-expression increased cell number compared with only miR-34a-5p over-expression ($p < 0.001$, Fig. 5c). In short, miR-34a-5p inhibited cell proliferation, migration and invasion through regulating the expression of JAG1.

miR-34a-5p Inhibited the Activity of Notch Pathway

In order to investigate the effect of miR-34a-5p on Notch1 pathway, we transfected miR-34a-5p mimics or miR-34a-5p inhibitors into HPV-HaCaT cells and assessed the expression of related proteins using western blotting. The results showed that the protein expression levels of JAG1, Notch1, NICD, Hes1 and Hey1 were decreased when miR-34a-5p over-

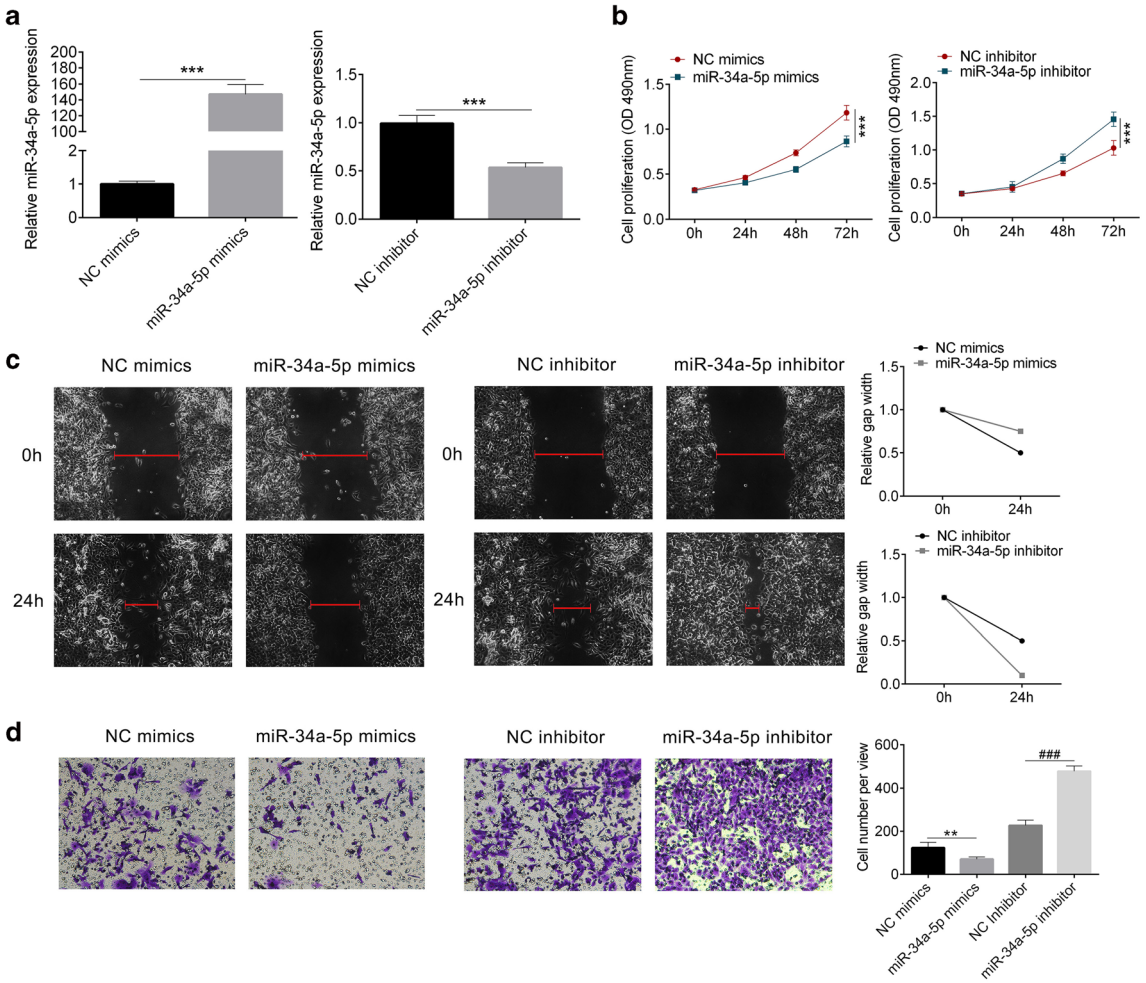

Fig. 2 miR-34a-5p inhibited cell viability, migration and invasion in HPV-HaCaT cells. a miR-34a-5p mimics, miR-34a-5p inhibitors, NC mimics or NC inhibitors were transfected into HPV-HaCaT cells using qRT-PCR. **b** Cell proliferation was detected using 3-(4, 5)-dimethylthiahiazo-(−z-y1)-3, 5-diphenytetrazoliumromide (MTT) assay. **c** Cell migration was detected using cell wound healing assay. **d** Cell invasion was detected using Transwell assay. $^{**}p < 0.01$; $^{***}p < 0.001$

expression ($p < 0.01$ or $p < 0.001$). Conversely, miR-34a-5p knock-down significantly up-regulated JAG1, Notch1, NICD, Hes1 and Hey1 expressions in HPV-HaCaT cells ($p < 0.05$, $p < 0.01$ or $p < 0.001$, Fig. 6). Therefore, the data indicated that miR-34a-5p could regulate the activity of Notch1 pathway.

Discussion

It is currently believed that the pathogen causing CA is HPV, among which HPV6, HPV11, HPV16, HPV18 are most closely related to CA, and then HPV16 is the most common type of HPV infection detected [22]. Ciotti et al. reported that HPV16E/E7 gene belongs to oncogene that induces malignant transformation of cells [23]. High-risk subtype HPV16E has been found to down-regulate miR-23b expression and induce cell migration in human cervical carcinoma [17]. Therefore, we learned from previous research and infected HPV16E6 gene into human keratinocyte HaCaT cells to establish CA cell models in vitro.

There is an article revealed that HPVE6 could down-regulate miR-34a and miR-34a expressions specifically targets p18Ink4c [24]. And in the absence of p63, the levels of miR-34a in primary keratinocytes and embryonic skin are elevated [25]. Therefore, we concluded that miR-34a-5p might be taken part in the regulation of HPV-infected CA. Kumar et al. showed that miR-34a is visibly down-regulated and inhibit cell proliferation, colony formation and migration in head and neck squamous cell carcinoma (HNSCC) [26]. Moreover, miR-34a over-expression inhibits HPV-positive cell viability in cervical cancer by targeting E2F3 [27]. It also has been found that miR-34a-5p expression level was lower in cervical intraepithelial neoplasia (CIN) compared with normal uterine epithelial cells, and was clearly decreased after HPV infection [28]. In the study, we detected the expression of miR-34a-5p in CA tissues and the effects on cell development in HPV-HaCaT cells. The results were consistent with

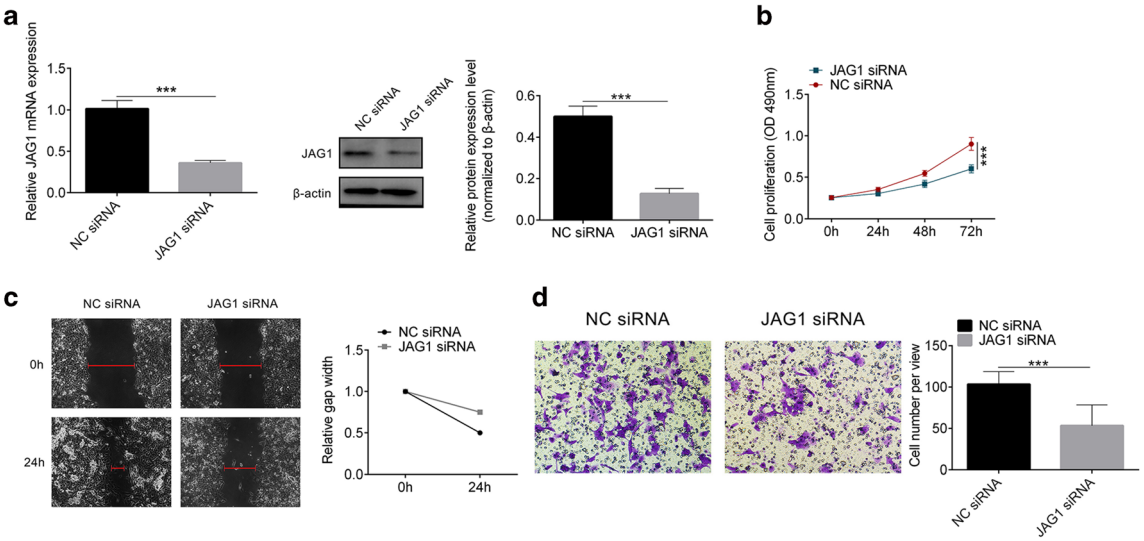

Fig. 3 JAG1 promoted cell viability, migration and invasion in HPV-HaCaT cells. a JAG1 siRNA or NC siRNA were transfected into HPV-HaCaT cells using qRT-PCR and western blotting. **b** Cell proliferation was detected using MTT assay. **c** Cell migration was detected using cell wound healing assay. **d** Cell invasion was detected using Transwell assay. ***$p < 0.001$

previous studies, that is, miR-34a-5p expression was lessened in CA tissues and HPV-HaCaT cells. miR-34a-5p overexpression inhibited cell proliferation, migration and invasion. We confirmed that miR-34a-5p is participated in the regulation of CA and influences cell development.

Furthermore, Hashimi et al. found that JAG1 3' UTR is the functional target of miR-34a. Meanwhile, JAG1 addition or miR-34a inhibition results in a decrease in key functions of immature dendritic cells (DCs) [29]. Pang et al. also reported that JAG1 is a target gene of miR-34a in cervical carcinoma and choriocarcinoma cells [20]. Therefore, we predicted the potential binding sites of miR-34a-5p and JAG1 by TargetScan, and verified that miR-34a-5p had a targeting effect on JAG1 using dual luciferase reporter gene assay. In

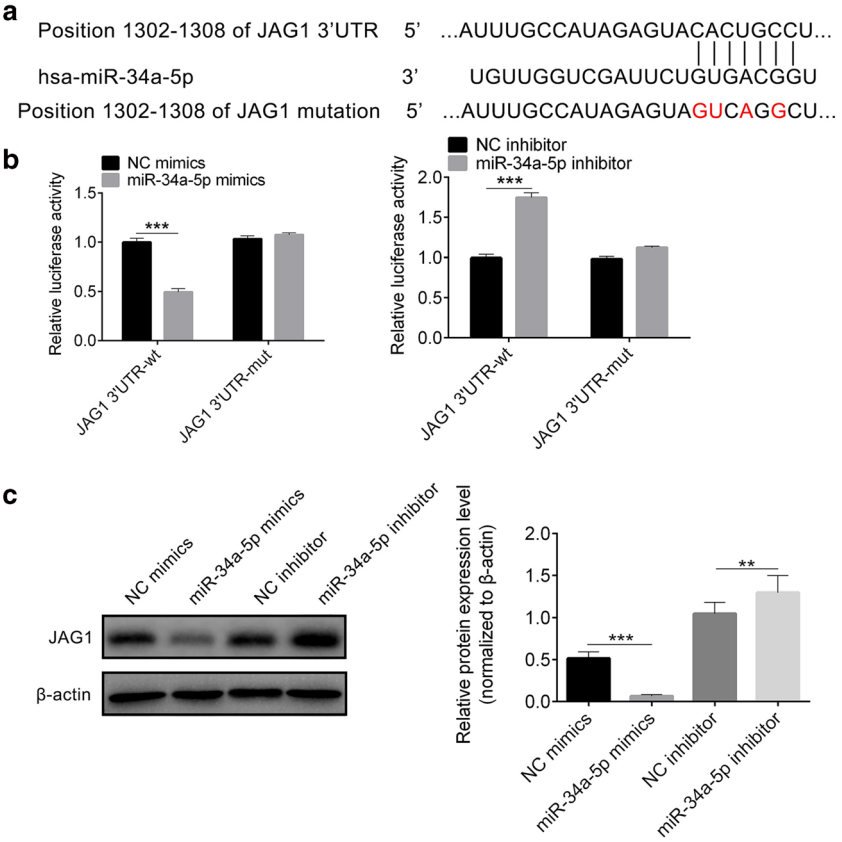

Fig. 4 miR-34a-5p had a targeting effect on JAG1. miR-34a-5p mimics, miR-34a-5p inhibitors, NC mimics or NC inhibitors were transfected into 293 T cells. a The potential binding sequence of miR-34a-5p and JAG1 was predicted by TargetScan (http://www.targetscan.org). **b** The targeting relationship between miR-34a-5p and JAG1 was determined by dual luciferase reporter gene assay. **c** JAG1 expression was detected using western blotting. **$p < 0.01$; ***$p < 0.001$

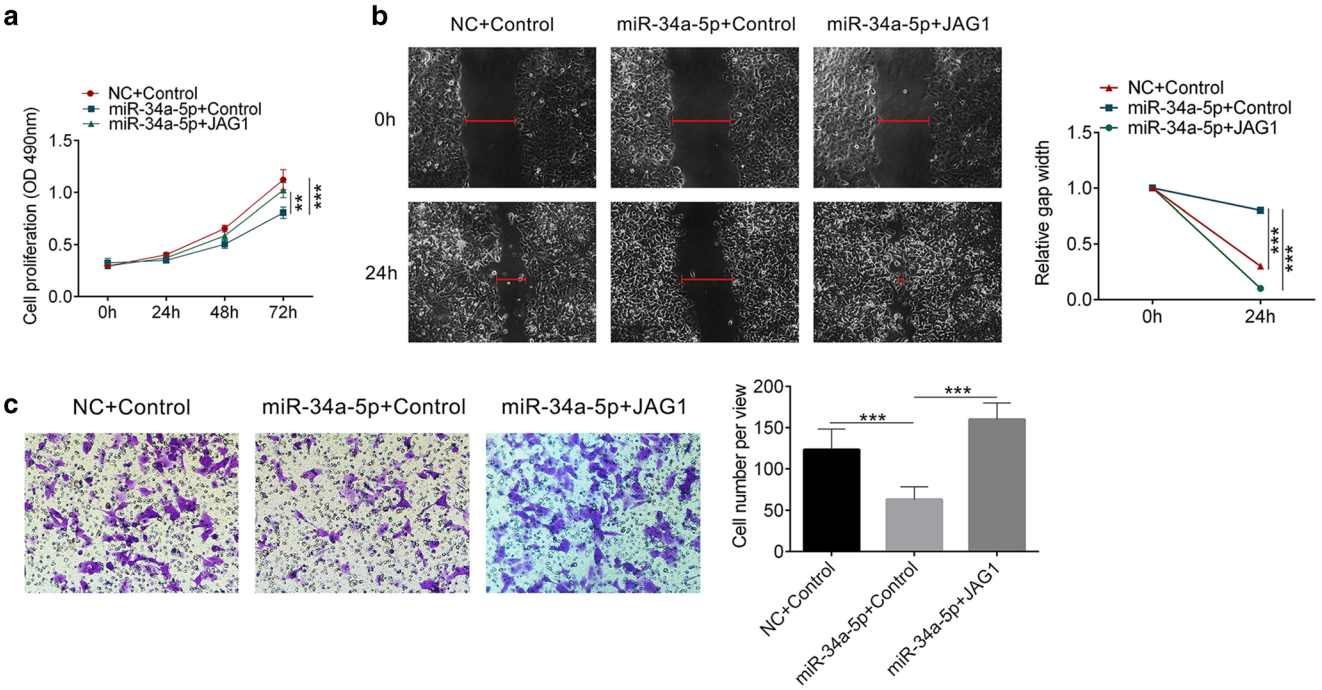

Fig. 5 miR-34a-5p inhibited cell development through inhibiting JAG1 expression. miR-34a-5p, JAG1, or NC were transfected into HPV-HaCaT cells. a Cell proliferation was detected using MTT assay. **c** Cell migration was detected using cell wound healing assay. **d** Cell invasion was detected using Transwell assay. $^{**}p < 0.01$; $^{***}p < 0.001$

addition, a study displayed that the mRNA and protein expression level of JAG1 are increased in adrenocortical carcinoma (ACC) [30]. The results were consistent with this research that the protein level of JAG1 was added in CA tissues and HPV-HaCaT cells. Additionally, JAG1 over-expression promoted cell development and alleviated the inhibitory effect of miR-34a-5p on HaCaT cells.

JAG1 is a ligand in the Notch pathway that enhances cell proliferation in a non-cellular autonomous manner by activating Notch signaling in adjacent cells [31]. The activation process of Notch pathway incudes the recognition and binding of receptors and ligands, the active digestion of receptors by two digestions, the transfer of active receptors to the nucleus and the activation of downstream effectors. It was also reported that the abnormal activation of Notch pathway is associated with the development and progression of tumors [30]. Notch1 is one of the target genes of miR-34a-5p, and is involved in the cellular gene regulation mechanisms, including cell fate determination, stem cell maintenance, cell proliferation, differentiation and survival [32]. Pang et al. reported in the literature that miR-34a inhibits cell invasion by regulating Notch pathway and its downstream matrix degrading enzymes [20]. The

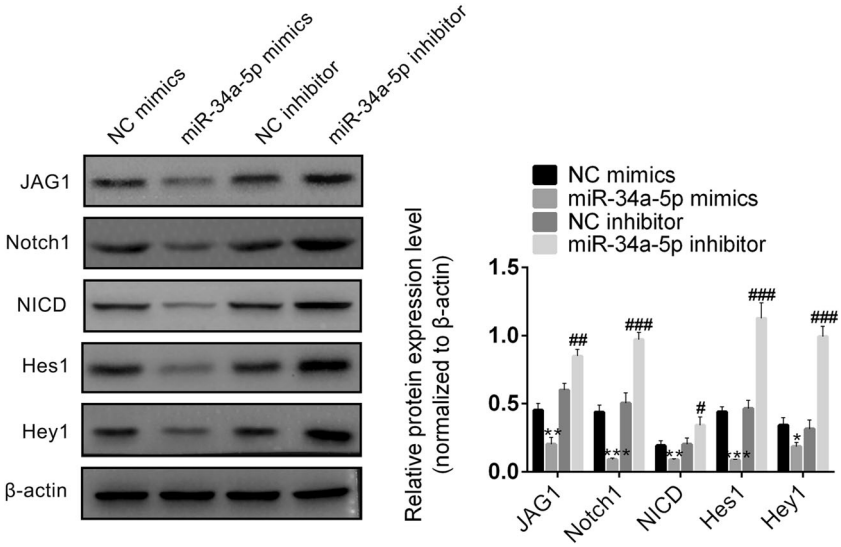

Fig. 6 miR-34a-5p inhibited the activity of Notch1 pathway. miR-34a-5p mimics, miR-34a-5p inhibitors, NC mimics or NC inhibitors were transfected into HPV-HaCaT cells. The protein expression levels of JAG1, Nothch1, NICD, Hes1 and Hey1 were detected using western blotting. $^{**}p < 0.01$, $^{***}p < 0.001$; * represented the comparison between miR-34a-5p mimics and NC mimics. $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$; # represented the comparison between miR-34a-5p inhibitor and NC inhibitor

results of this study suggested that the expressions of downstream genes NICD, Hes1 and Hey1, as well as Notch1 were decreased in Notch pathway when miR-34a-5p was overexpressed. These results suggested that miRNA-34a-5p is involved in the regulation of Notch1, and indeed inhibits the activity of Notch pathway in HPV-infected HaCaT cells, resulting in a reduction in downstream protein expression.

Conclusion

In conclusion, miR-34a-5p/JAG1/Notch1 regulatory network could inhibit cell proliferation, migration and invasion of HPV-infected keratinocytes. These results provide a new research direction for the pathogenesis of CA induced by HPV. Moreover, the next step will be to verify and discover the target genes and mechanisms of miR-34a-5p through more experiments.

Authors' Contributions YG and TY conceived and designed the experiments, MY, LLW and XFL analyzed and interpreted the results of the experiments, FW and YLH performed the experiments.

Data Availability All data generated or analyzed during this study are included in this published article.

Compliance with Ethical Standards

Competing Interests The authors declare that they have no competing interests, and all authors should confirm its accuracy.

Ethics Approval and Consent to Participate The animal use peotocol listed below has been reviewde and approved by the Animal Ethical and Welfaer Committee.

Patient Consent for Publication Not Applicable.

Informed Consent Written informed consent was obtained from a legally authorized representative(s) for anonymized patient information to be published in this article.

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