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Research Article

GMNN and RFC4 Proteins Synergistically Regulate the Proliferation and Invasion of Uterine Corpus Endometrial Carcinoma Cells Via the Notch Pathway

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Abstract

Objective: This study investigated the interaction of GMNN and RFC4 proteins with the Notch pathway and their effects on the proliferation and invasion of Uterine Corpus Endometrial Carcinoma (UCEC) cells.

Materials and Methods: Pan-cancer expression data of GMNN and RFC4 were obtained from databases to analyze their differential expression in UCEC tissues and their correlation with histological grading and patient survival. Ishiwaka cells were cultured in vitro and transfected with GMNN-RFC4-short hairpin RNA (shRNA), GMNN-shRNA, and RFC4-shRNA. Transwell, scratch, and colony formation assays were conducted to detect UCEC cell invasion, migration, and proliferation, respectively. GMNN, RFC4, and Notch1 protein expression in UCEC cells was measured with western blotting. GMNN, RFC4, and Notch1 expression in UCEC tissues of different differentiation degrees was examined with immunohistochemistry to analyze their relationship with the clinicopathological characteristics of patients.

Results: Database analysis revealed that GMNN and RFC4 expression was high in UCEC tissues, which was positively correlated with histological grading and negatively correlated with the overall survival of patients. GMNN, RFC4, and Notch1 protein expression was significantly diminished in Ishiwaka cells after silencing of GMNN and RFC4 alone or in combination. Ishiwaka cell proliferation, invasion, and migration were markedly decreased by silencing GMNN or RFC4 ($P<0.05$), and these trends were further promoted by silencing both GMNN and RFC4. Moreover, these three proteins were expressed at lower levels with higher differentiation levels of UCEC tissues.

Conclusion: Silencing of GMNN and RFC4 proteins alone or in combination reduces Notch1 protein decreases and represses the malignant biological behaviors of UCEC cells, indicating GMNN and RFC4 proteins as highly promising new molecular indicators for the pathological diagnosis and treatment of UCEC in the future.

Keywords: Uterine corpus endometrial carcinoma; Notch pathway; Protein expression; Malignant biological behaviors

Introduction

Uterine Corpus Endometrial Carcinoma (UCEC) is an epithelial malignancy of the endometrium [1], whose incidence ranks first among malignant tumors of the female reproductive tract in developed countries and some developed regions of China [2,3]. Although the 5-year survival rate of UCEC patients is up to about 76%, patients developing distant metastasis have a poor prognosis, with a 5-year survival rate of only 17% [4,5]. Therefore, it is valuable to explore the mechanism of invasion and metastasis in UCEC for improving the prognosis of patients. In The Cancer Genome Atlas (TCGA) database, GMNN and RFC4 ex-

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pression was observed to be significantly higher in UCEC tissues than in normal tissues. As reported, the invasion and metastasis of malignant tumors are regulated by various pathways, and the Notch pathway is involved in the physiological and pathological processes of most biological cells, such as proliferation and metastasis. Prior studies demonstrated that GMNN influenced the biological behaviors of cells through the Notch pathway [6] and that RFC4 was involved in Notch signaling transduction [7]. In this context, this study probed the correlation of GMNN and RFC4 with the Notch pathway in UCEC cells and the correlation between their expression and UCEC cell proliferation and invasion, which preliminarily analyzed the mechanism of GMNN and RFC4 in UCEC and provided a theoretical basis for the treatment and prognosis evaluation of UCEC.

Materials and Methods

Participants

This study involved paraffin-embedded blocks of cancer tissues from 147 UCEC patients admitted to Binhai County People's Hospital between June 2014 and June 2022. These patients were aged 35-72 (48.71 ± 2.16) years and consisted of 64 menopausal patients and 83 non-menopausal patients. Among the patients, the initial lesion size ranged from 1 to 8 (4.21 ± 0.77) cm, and there were 30 patients with the initial lesion size of < 2 cm, 26 patients with the size of 2−3 cm, 20 patients with the size of 3−4 cm, 24 patients with the size of 4−5cm, and 47 patients with the size of > 5 cm. Lymph node metastasis was observed in 62 patients and non-metastasis in 85 patients. In addition, the latest Thinprep Cytology Test results prior to surgery were collected to analyze the inflammatory status of patients. All procedures were performed in compliance with relevant laws and institutional guidelines and have been approved by the binhai county people's hospital (2301147). And informed consent was obtained for experimentation with patients.

Cell line and Main Reagents

The following cell line and reagents were used: Ishiwaka cells (a human UCEC cell line; American Type Culture Collection, Manassas, VA, USA), high-glucose Dulbecco's Modified Eagle Medium (DMEM; SH30023; HyClone, Logan, UT, USA), fetal bovine serum (FBS; 141215; Tianhang Biotechnology Co., Ltd., Zhejiang, China), consumables (Solarbio, Beijing, China) related to cell culture and experiments including dimethyl sulfoxide, penicillin-streptomycin, and Matrigel, GMNN-short hairpin RNA (shRNA) and RFC4-shRNA lentiviruses and negative control (GenePharma, Shanghai, China), 3 μm Transwell chambers (3378; Corning Company, Corning, NY, USA), crystal violet staining solutions (AS1086; ASPEN, Wuhan, China), a sodium dodecyl-sulfate polyacrylamide gel electrophoresis gel preparation kit (AS1012; ASPEN), Radio-Immunoprecipitation Assay (RIPA) protein lysis (AS1004; ASPEN), Tween-20 (AS1100; ASPEN), a bicinchoninic acid kit (AS1086; ASPEN), Exposable Protein Marker (DM211; TransGen Biotech, Beijing, China), polyvinylidene fluoride membranes with a pore size of 0.45 μm (IPVH00010; Millipore, Billerica, MA, USA), rabbit-derived primary antibodies against RFC4 (ab96852; Abcam, Cambridge, UK), Notch1 (ab52627; Abcam), and GMNN (ab195047; Abcam).

Methods

Bioinformatics analysis: Correlations between GMNN and RFC4 expression and clinical data were analyzed with TCGA (https://portal.gdc.cancer.gov/), Gene Expression Profiling Interactive Analysis 2 (GEPIA2; http://gepia.cancer-pku.cn/detail.php), and Kaplan-Meier Plotter (http://kmplot.com/analysis/).

Cell grouping: Cells were arranged into four groups: the blank control group (the A group): Ishiwaka cells were added with an equal amount of Phosphate-Buffered Saline (PBS); the GMNN and RFC4 silencing group (the B group): Ishiwaka cells were transfected with both GMNN-shRNA and RFC4-shRNA lentiviruses; the GMNN silencing group (the C group): Ishiwaka cells were transfected with GMNN-shRNA lentiviruses; the RFC4 silencing group (the D group): Ishiwaka cells were transfected with RFC4-shRNA lentiviruses.

Cell culture: Cells were cultured with the freshly prepared complete DMEM containing 10% FBS, 1% penicillin-streptomycin, and 0.005 mg/mL insulin in a constant-temperature incubator at 37℃ with 5% CO_2. Then, the medium was renewed every 2 days, and cells were passaged after the cell confluence reached 80%.

Lentivirus transfection and transfection efficiency detection: According to the lentivirus transfection manual, target cells with favorable growth status were selected 24 h before transfection and added to 24-well plates ([2−10] × 10^4 cells/well) for culture under routine culture conditions. When the cell confluence reached 80% after 24 h, the medium was aspirated, and each well was added with 0.5 mL of diluted lentivirus suspensions. Meanwhile, the negative control cell line was constructed. Cells were routinely cultured at 37°C in a 5% CO_2 incubator. The cell growth status was observed 12 h after transfection, and the medium was replaced with a conventional medium approximately 48 h later. Green fluorescent protein expression in cells was observed under an inverted phase-contrast fluorescence microscope at 96 h post-transfection, and GMNN and RFC4 protein expression in Ishiwaka cells was measured with western blotting after lentivirus transfection.

Detection of cell invasion, migration, and proliferation: Transwell assay was conducted to test Ishiwaka cell invasion. Specifically, Matrigel was pre-coated in the apical chamber of the Transwell chamber on ice and fully solidified in the incubator. Cells were resuspended with a serum-free DMEM, and then the suspensions were seeded in the apical chamber at a density of 3 × 10^4 cells/μL (200 μL suspensions per well). The basolateral chamber of the Transwell chamber was added with a serum-free DMEM (600 μL/well). After 24 h of incubation in the incubator, the cells were fixed with paraformaldehyde, stained with crystal violet staining solutions, and gently rinsed with running water. Cells that did not penetrate the membrane were wiped off with a cotton swab, and cells that penetrated the membrane were photographed and counted under an inverted microscope.

Scratch assay was performed to examine Ishiwaka cell migration. In detail, Ishiwaka cells were cultured in Petri dishes. After cells reached 80% confluence, a solid line was drawn with a marker pen along a straightedge on the back of the Petri dish, and then at least 5 straight lines were drawn perpendicular to the solid line with a toothpick. The cells were rinsed with PBS to remove detached cells and photographed under the microscope. After 24 h of culture, cells were photographed under the microscope again.

Colony formation assay was used to measure Ishiwaka cell proliferation. In brief, single Ishiwaka cells were separated, seeded on a Petri dish, and added with a complete medium. The medium was renewed once every three days, and the cul-

ture was continued for 15 days. After the formation of cell colonies, colonies were observed, photographed, and counted. The assay was repeated three times, and the results of the three experiments were averaged.

Western blotting: Cells were lysed with RIPA lysis solutions to extract the total protein, and an equal amount of protein samples from each group was subjected to electrophoresis and transferred onto membranes. Next, the membranes were incubated with rabbit-derived primary antibodies (1:1000) against RFC4, GMNN, Notch1, and glyceraldehyde 3-phosphate dehydrogenase overnight and washed, followed by 2 h of incubation with horseradish peroxidase-labeled goat anti-rabbit immunoglobulin G (1:10,000). The membrane was washed and then exposed and developed with an ultrasensitive enhanced chemiluminescence kit.

Immunohistochemistry: This study involved paraffin-embedded blocks of cancer tissues from 147 UCEC patients admitted to Binhai County People's Hospital between June 2014 and June 2022. These patients were aged 35-72 (48.71 ± 2.16) years and consisted of 64 menopausal patients and 83 non-menopausal patients. Among the patients, the initial lesion size ranged from 1 to 8 (4.21 ± 0.77) cm, and there were 30 patients with the initial lesion size of < 2 cm, 26 patients with the size of 2–3 cm, 20 patients with the size of 3–4 cm, 24 patients with the size of 4–5cm, and 47 patients with the size of > 5 cm. Lymph node metastasis was observed in 62 patients and non-metastasis in 85 patients. In addition, the latest Thinprep Cytology Test results prior to surgery were collected to analyze the inflammatory status of patients.

Inclusion criteria for patients were as follows: (1) patients who were diagnosed with UCEC by two or more pathologists; (2) patients who had complete pathological data and unobstructed telephone and met the conditions for follow-up; (3) patients with well-preserved paraffin-embedded blocks for immunohistochemistry. Immunohistochemistry was utilized to detect the expression of three proteins, GMNN, RFC4, and Notch1, in the paraffin-embedded blocks. The experiment was approved by the Ethics Committee of Binhai County People's Hospital, and all patients gave informed consent.

Statistical Methods

Qualitative data were analyzed with the chi-square test, while quantitative data were analyzed with the *u*-test and *t*-test. Logistic regression was used to analyze correlations. All data were analyzed with SPSS22.0 statistical software. Differences were statistically significant at $P<0.05$.

Results

Correlations between GMNN and RFC4 Protein Expression and the Clinical Data of Patients

The analysis with the TCGA database demonstrated that GMNN and RFC4 expression was markedly higher in UCEC tissues than in normal tissues (Figure 1A, B).

It was observed in the GEPIA2 database that GMNN and RFC4 expression was significantly different between normal and UCEC tissues (Figure 1C, D) and that their expression was positively correlated with histological grading (Figure 1E, F).

The Kaplan-Meier Plotter showed that GMNN and RFC4 expression was negatively correlated with the overall survival of UCEC patients (Figure 1G-J).

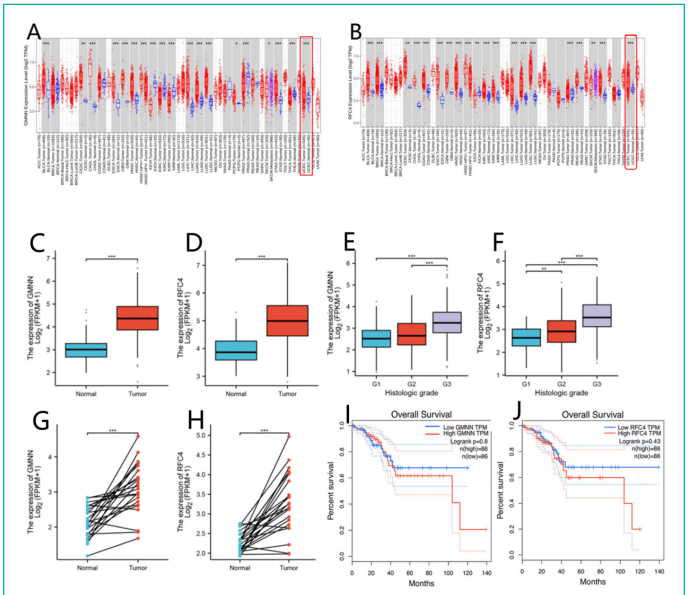

Figure 1: Correlations of GMNN and RFC4 protein expression with the clinical data of patients.
Note: A: GMNN expression in normal and UCEC tissues analyzed with the TCGA database. B: RFC4 expression in normal and UCEC tissues analyzed with the TCGA database. C-D: Difference in GMNN and RFC4 expression in normal and UCEC tissues in the GEPIA2 database. E-F: Relationship between GMNN and RFC4 expression and the histological grading of UCEC analyzed with the GEPIA2 database. G-H: Differential expression of GMNN and RFC4 in normal and UCEC tissues. I-J: Relationship between GMNN and RFC4 expression and the overall survival of UCEC analyzed with the Kaplan-Meier Plotter.

Table 1: Comparisons of general information between the two groups (cases/%).

Items	Moderate-to-high differentiation group (n=69)	Low differentiation group (n=78)	χ^2	*P*
Age				
≥ 50	37 (53.62)	32 (41.03)	0.026	0.416
< 50	32 (46.37)	46 (58.97)		
Menopause				
Yes	27 (39.13)	37 (47.44)	0.116	0.719
No	42 (60.87)	41 (52.56)		
Delivery history				
Yes	51 (73.91)	62 (79.49)	0.057	0.265
No	18 (26.09)	16 (20.51)		
History of hypertension				
Yes	16 (23.19)	14 (17.95)	0.216	0.527
No	53 (76.81)	64 (82.05)		
BMI				
≥ 28 kg/m^2	31 (44.93)	46 (58.97)	0.839	0.361
< 28 kg/m^2	38 (55.07)	32 (41.03)		

Table 2: Analysis of clinicopathologic characteristics of patients in the two groups (cases/%).

Items	Moderate-to-high differentiation group (n = 69)	Low differentiation group (n = 78)	χ″	*P*
Tumor size				
< 5	45 (65.22)	23 (29.49)	7.178	0.003
≥ 5	24 (34.78)	55 (70.51)		
Lymph node metastasis				
Negative	49 (71.01)	36 (46.15)	6.482	0.016
Positive	20 (28.99)	42 (53.85)		
Myometrial invasion				
≥ 1/2	14 (20.29)	52 (66.67)	15.364	< 0.001
< 1/2	55 (79.71)	26 (33.33)		
Inflammation				
Severe inflammation	16 (23.19)	51 (65.38)	17.728	< 0.001
Mild-to-moderate inflammation	53 (76.81)	27 (34.62)		
GMNN expression				
Positive	41 (59.42)	65 (83.33)	9.426	< 0.001
Negative	28 (40.58)	13 (16.67)		
RFC4 expression				
Positive	32 (46.38)	63 (80.77)	9.774	< 0.001
Negative	37 (53.62)	15 (19.23)		
Notch1 expression				
Positive	42 (60.87)	70 (89.74)	19.907	< 0.001
Negative	27 (39.13)	2 (2.56)		

GMNN, RFC4, and Notch1 Protein Expression in UCEC cells after Lentivirus Transfection

After GMNN-shRNA and RFC4-shRNA lentiviruses were respectively introduced into Ishiwaka cells, GMNN and RFC4 protein expression was detected to evaluate transfection efficiency (Figure 2). Furthermore, Ishiwaka cells were transfected with GMNN-shRNA and RFC4-shRNA lentiviruses alone or in combination, followed by the measurement of GMNN, RFC4, and Notch1 protein expression with western blotting (Figure 3A). The results revealed that GMNN, RFC4, and Notch1 protein expression in the B group was statistically significantly decreased compared with that in the A group ($P < 0.05$), suggesting that lentivirus transfection successfully silenced GMNN and RFC4, and the silencing of both GMNN and RFC4 reduced Notch1 protein expression, therefore blocking the Notch pathway. The protein expression of RFC4, GMNN, and Notch1, particularly GMNN, was statistically markedly lower in the C group than in the A group ($P < 0.05$), illustrating that the GMNN-shRNA lentivirus was successfully transfected into Ishiwaka cells and that GMNN silencing lowered RFC4 and Notch1 protein expression. Moreover, RFC4, GMNN, and Notch1 protein expression in the C group was insignificantly different from that in the B and D group ($P > 0.05$). This result indicated that the silencing of GMNN and RFC4 alone and in combination exerted similar effects, highlighting that GMNN and RFC4 might synergistically work.

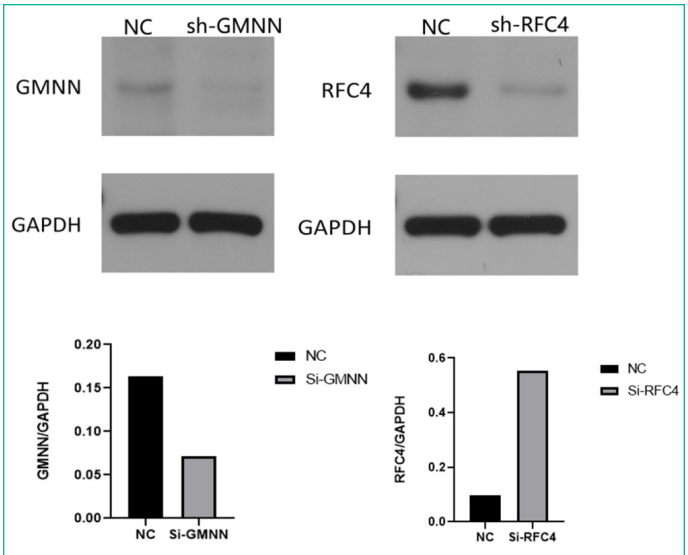

Figure 2: Efficiency of lentivirus transfection.

The protein expression of RFC4, GMNN, and Notch1, especially RFC4, was statistically significantly lower in the D group than in the A group ($P < 0.05$), indicating the successful transfection of the RFC4-shRNA lentivirus and that RFC4 silencing declined GMNN and Notch1 protein expression. Meanwhile, RFC4, GMNN, and Notch1 protein expression in the D group was insignificantly different from that in the B and C group ($P > 0.05$).

UCEC cell Invasion and Migration after Lentivirus Transfection

Transwell assay results unveiled that the number of cells penetrating the chamber membrane within 24 h was lower in the B (22.45 ± 2.24), C (107.95 ± 5.43), and D (112.55 ± 6.34) group than in the A (162.75 ± 6.15) ($P < 0.05$) (Figure 3B). Scratch assay results revealed that the wound healing rate of cells was lower in the B (12.06% ± 1.15%), C (30.55% ± 2.13%), and D (28.88% ± 2.67%) groups than in the A group (53.99% ± 3.56%) ($P < 0.05$) (Figure 3C).

The results illustrated that simultaneous silencing of GMNN and RFC4 more significantly reduced UCEC cell invasion and migration than silencing GMNN and RFC4 alone, implying that GMNN and RFC4 proteins synergistically lower UCEC cell invasion and migration.

UCEC Cell Proliferation after Lentivirus Transfection

According to colony formation assay results, the B (10.53 ± 1.36), C (29.60 ± 1.59), and D (27.80 ± 2.31) groups had a lower rate of colony formation than group A (44.80 ± 2.69) ($P < 0.05$) (Figure 3D).

The results suggested that simultaneous silencing of GMNN

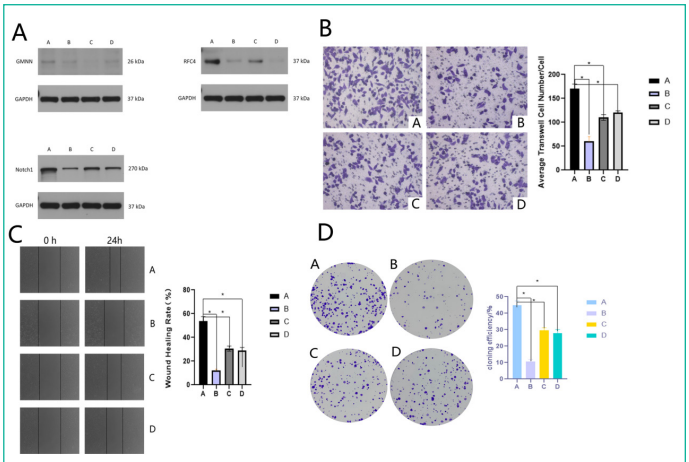

Figure 3: GMNN, RFC4, and Notch1 protein expression, proliferation, invasion, and migration in UCEC cells after lentivirus transfection.

Note: A: GMNN, RFC4, and Notch1 protein expression measured with western blotting. B: Ishiwaka cell invasion detected with Transwell assay ($x^2 \pm S$; n = 4). C: Ishiwaka cell migration tested with scratch assay ($x^2 \pm S$; n = 4). D: Ishiwaka cell proliferation examined with colony formation assay ($x^2 \pm S$; n = 4). A: the blank control group; B: the GMNN-shRNA + RFC4-shRNA group; C: the GMNN-shRNA group; D: the RFC4-shRNA group.

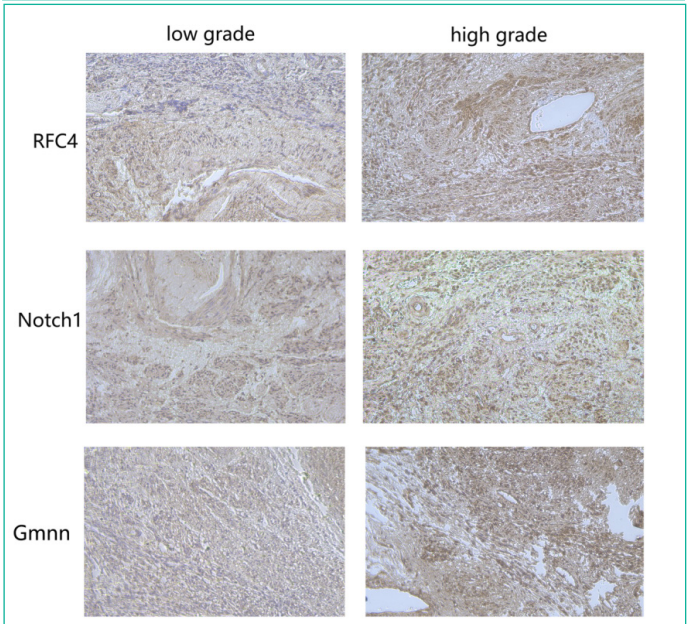

Figure 4: GMNN, RFC4, and Notch1 protein expression in paraffin-embedded tissue blocks detected with immunohistochemistry (200X).

and RFC4 more significantly diminished UCEC cell proliferation than silencing GMNN and RFC4 alone, highlighting that GMNN and RFC4 proteins synergistically repress UCEC cell proliferation.

GMNN, RFC4, and Notch1 Protein Expression in Paraffin-Embedded Tissue Blocks

Based on the pathological diagnosis results, the collected paraffin-embedded tissue blocks were classified into low differentiation (78 cases) and moderate-to-high differentiation (69 cases) groups according to the degree of differentiation. General information of patients was not statistically significantly different between the two groups ($P > 0.05$), confirming that the two groups were comparable (Table 1). Then, immunohistochemistry was carried out to detect GMNN, RFC4, and Notch1 protein expression in the two groups and to analyze the relationship between their protein expression and the clinicopathological features of patients (Table 2).

According to the clinicopathological data of patients, significant differences were observed between the two groups in terms of tumor size, lymph node metastasis, myometrial invasion, and inflammation ($P < 0.05$). To be specific, compared with the moderate-to-high differentiation group, the low differentiation group had a higher percentage of patients with tumors larger than 5 cm, patients with positive lymph node metastasis, patients with myometrial invasion of more than 1/2, and patients with severe inflammation ($P < 0.05$), reflecting a worse prognosis of patients.

GMNN, RFC4, and Notch1 proteins were mainly localized in the nucleus and cytoplasm. Immunohistochemistry was conducted with the EnVision method (Figure 4), which unraveled that the positive rates of the three proteins were substantially higher in the low differentiation group than in the moderate-to-high differentiation group. This result suggested that GMNN, RFC4, and Notch1 expression was positively correlated with the malignancy degree of UCEC, highlighting GMNN, RFC4, and Notch1 as potential target genes for the treatment and diagnosis of UCEC.

Discussion

UCEC typically affects perimenopausal and postmenopausal elderly women [8], which has become the most frequent malignant tumor of the reproductive tract in elderly women due to its increasing incidence in recent years with the development of population aging [9]. Currently, the main treatment for UCEC is surgical resection supplemented by radiotherapy and drug therapy. Unfortunately, most patients have a short survival time and poor quality of life and are highly susceptible to drug resistance after surgery. The rapid development of targeted therapies has provided new treatment for UCEC. Nevertheless, most targeted agents are still in the trial stage. Accordingly, the search for targeted molecular markers is key to developing novel treatments for UCEC. Studies on GMNN are scarce, focusing mainly on its role in biological development and rarely on its role in tumors [10]. Therefore, although there are some clinical trials revealing that GMNN expression is proportional to the malignancy degree of tumors, few studies have been conducted on its mechanism. A basic study elucidated that the expression of GEMC1, a member of the GMNN protein family, declined when Notch was activated [11], implicating that GMNN may function through the Notch pathway. The Notch pathway is an important signaling system capable of regulating metazoan development and adult tissue homeostasis and playing a role in a wide range of events including proliferation, apoptosis, and boundary formation and cell fate determination [12]. In different kinds of cells, Notch signaling can suppress or facilitate cell differentiation and thus influence cell proliferation and invasion. Of note, a study on UCEC elaborated that the activation of the Notch pathway enhanced UCEC cell proliferation and metastasis [13].

RFC4 is one of several subunits of the Replication Factor C (RFC) complex, which functions as a polymerase accessory protein in DNA replication and repair [14]. Importantly, a prior study revealed that the blockade of the Notch1 pathway substantially diminished RFC4 expression in cells [15]. Moreover, RFC4 dysregulation promotes cell proliferation and tumorigenesis. For instance, a former study detected significantly increased RFC4 expression in 81 out of 105 lung cancer tissues [16]. Another study demonstrated that RFC4 overexpression enhanced invasion and self-renewal of non-small cell lung cancer, whereas RFC4 silencing greatly decreased invasion and self-renewal of cells [17]. In addition, this study also displayed that silencing

of RFC4 substantially inhibited the ability of lung cancer cells to form pulmonary metastatic tumors or subcutaneous tumors in mice, indicating significant promoting effects of RFC4 protein on tumor metastasis and invasion. Furthermore, some relevant clinical trials showed that the metastasis of RFC4-overexpressing cells was markedly increased in various organs and tissues, especially in the brain and bone [18].

Recent basic studies have reported that GMNN modulates the Notch pathway to control somite segmentation during somite formation [19]. Additionally, RFC4 was also revealed to play a pivotal role in the Notch pathway [20]. Therefore, it is hypothesized that GMNN and RFC4 may synergistically mediate the Notch pathway to influence the progression of UCEC. Notch1 protein acts as not only an agonist of the Notch pathway but also a marker of Notch pathway activation, and its upregulation reflects Notch pathway activation.

GMNN and RFC4 expression in UCEC tissues and their relationship with the prognosis of patients are seldom reported. In the present study, UCEC cells (Ishiwaka) were cultured in vitro, and GMNN and RFC4 were silenced in cells using lentivirus. The results showed a decrease in proliferation, invasion, and migration of UCEC cells after GMNN and RFC4 silencing, illustrating that silencing of GMNN and RFC4 impeded UCEC cell proliferation, invasion, and migration. Further, GMNN, RFC4, and Notch1 protein expression was examined with western blotting, which presented that GMNN silencing alone resulted in a marked reduction in RFC4 and Notch1 protein expression, while RFC4 silencing alone obviously lowered GMNN and Notch1 protein expression. It was also found that simultaneous silencing of GMNN and RFC4 more significantly diminished GMNN, RFC4, and Notch1 protein expression than silencing of GMNN and RFC4 alone. These results indicated that GMNN and RFC4 might activate the Notch pathway by binding to each other, thus facilitating the metastasis and proliferation of UCEC. In summary, GMNN and RFC4 are strongly related to the malignant biological behaviors of UCEC, and there is a positive correlation between GMNN and RFC4 expression. Moreover, GMNN and RFC4 synergistically promote the malignant biological behaviors of UCEC by activating the Notch pathway. This study illustrates the high potential of GMNN and RFC4 as new molecular indicators for the pathological diagnosis and treatment of UCEC in the future.

Author Statements

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