Original Article

Expression of combined interference of slug and FoxC2 in endometrial carcinoma and its clinicopathological relationship

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Background: Endometrial carcinoma is one of the three major malignant tumors in female reproductive system. The current research aimed to investigate the relationship between the Slug and FoxC2 expression and the proliferation, invasion and metastasis of endometrial carcinoma.

Methods: The expression of *Slug* and *FoxC2* genes between 124 endometrial carcinoma tissues and 35 normal endometrial tissues was analyzed through immunohistochemistry. The endometrial carcinoma cell lines Ishikawa and RL-952 were cultured, the Slug-shRNA and FoxC2-shRNA expression vectors were constructed, and the endometrial carcinoma cells interfering with the expression of *Slug* and *FoxC2* genes were also established. Western blotting and RT-PCR were employed to verify whether shRNA could down regulate the expression of *Slug* and *FoxC2* genes. Additionally, the proliferation, migration and invasion capacities in both cell lines after interfering with Slug and FoxC2 was detected through CCK-8 and Transwell assay respectively. Furthermore, MMP2 and MMP9 were detected by ELISA and epithelial-mesenchymal transition (EMT) related proteins including E-cadherin, N-cadherin and Vimentin were assessed by Western blotting analysis.

Results: Compared with normal endometrial tissues, the Slug and FoxC2 expression levels in endometrial carcinoma tissues were remarkably increased. shRNAs transfection significantly down-regulated expressions in both endometrial carcinoma cell lines. The proliferation, invasion and migration ability were significantly inhibited by Slug-shRNA and FoxC2-shRNA compared with the control group. The expression of E-cadherin was increased while the expression of N-cadherin, Vimentin, MMP-2 and MMP-9 was suppressed by the Slug-shRNA and FoxC2-shRNA.

Conclusions: Slug and FoxC2 could be used as a prognostic factor of endometrial carcinoma. Interfering with the expression of Slug and FoxC2 in endometrial carcinoma cell lines could effectively inhibit the proliferation, invasion and migration, and its mechanism is related to the inhibition of EMT. Slug and FoxC2 are potential targets for the treatment of endometrial carcinoma.

Keywords: Endometrial carcinoma; gene targeting regulation; Slug; FoxC2; epithelial-mesenchymal transition (EMT)

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Introduction

Endometrial carcinoma is one of the most malignant tumors in female reproductive system, which has severely damaged the life health in women. However, there is still lacking effective prognostic indicators and treatment options for the clinical treatments. Slug is a transcription factor of SNAIL family that can encode the zinc finger protein, which is reported to be up-regulated in multiple malignant tumors. FoxC2 is a member of the Fox protein family, which can regulate the expression of a series of downstream genes, and participate in the epithelial-mesenchymal transition (EMT) (1-3). Transcription factors Slug and FoxC2 have been reported to play important roles in tumor invasion and metastasis, which are the important regulatory molecules during tumor cell migration and metastasis (4).

Short hairpin RNA (shRNA) is a kind of RNA sequence that can form a tight hairpin turn, which can silence gene expression. The double-strand small molecule RNA or shRNA has been utilized in clinical test to treat several diseases, and certain achievements have been attained in terms of tumor treatment. In the current study, shRNAs were used to down-regulate the Slug and FoxC2 expression in Ishikawa and RL-952 endometrial carcinoma cell lines, and cell proliferation, migration and invasion were further evaluated. Then we tested the relevant indicators of EMT after transfection to gain a deeper understanding of the role of Slug and FoxC2 in endometrial carcinoma.

We present the following article in accordance with the MDAR checklist (available at http://dx.doi.org/10.21037/tcr-20-809).

Methods

Patients

The endometrial carcinoma tissues were retrospectively collected from 124 patients in the Pathology Department of the First Affiliated Hospital of Bengbu Medical College from January 2014 to December 2015. Before surgery, all endometrial carcinoma patients did not receive any anticancer treatments, including chemotherapy and radiotherapy. Patients with endometrial carcinoma were confirmed by pathologic diagnosis. The normal endometrial tissues were collected into the control group, which were derived from 35 patients receiving endometrial tissue biopsy and hysterectomy due to other benign disease in Gynecology.

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by institutional ethics board of The First Affiliated Hospital of Bengbu Medical College (No. BBMEC-2018-10) and informed consent was taken from all the patients.

Immunohistochemical staining

Endometrial tissues resected from uterus of patients, were rinsed with normal saline, fixed with 4% formalin for 24 h, dehydrated and embedded. The samples were sliced into sections 0.4 μm in thickness, followed by dewaxing of the sections and antigen retrieval in citric acid solution for 3 min at 121 ℃. After natural cooling, the sections were treated with 3% H_2O_2 solution for 10 min, followed by the addition of anti-Slug (ab180714, Abcam, Cambridge, MA, USA) and anti-FoxC2 (ab5060, Abcam, Cambridge, MA, USA) rabbit anti-human primary antibodies to incubate for 1 h at 60 ℃, rinsing with PBS for 3×5 min and incubation with secondary antibody for 30 min at 37 ℃. Then, the sections were washed with PBS, followed by DAB staining for visualization, nuclear counterstaining of hematoxylin and microscopic observation. The Image-Pro Plus software was adopted for image analysis by another person who was blind to the group assignments, and the integrated optical density/area ratio was used as the criterion to determine the protein expression contents of Slug and FoxC2.

Cell culture and transfection

Human endometrial carcinoma cell lines Ishikawa (catalog number BH-X5152, Shanghai Bohu Biotechnology Co., Ltd.) and RL-952 (catalog number BNN1364, Shanghai Beinuo Biology Co., Ltd.) were maintained in RPMI 1640 medium (HyClone, Thermo Scientific) supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 U/mL streptomycin, and incubated in a humidified incubator under the conditions of 5% CO_2 and 95% air at 37 ℃. Subsequently, Ishikawa and RL-952 cells at logarithmic growth phase were collected and seeded randomly into a 96-well plate (2×10^4 cells/mL) and cultured for 24 h. The 2–5 generations cells were used for the following experiments.

The target plasmid DNA and Lipofectamine 2000 were diluted in the serum-free Opti-MEM. Cells at the density of 60–70% were collected and incubated with the mixed medium for 20 min at room temperature. Then, the mixtures were added to the 96-well plate containing both cells and culture solution, agitated gently, and placed in the

incubator. The Slug-shRNA and FoxC2-shRNA expression vector (GenePharma, Shanghai, China) treatment groups (containing cells, complete medium, Slug-shRNA and FoxC2-shRNA), negative control group (containing cells, complete medium, and negative control-shRNA), and blank control group (containing cells and complete medium) were set, and 4 duplicates were prepared for each group.

Protein expression changes detected by Western blotting

Cells were harvested at 48 h after transfection, and lysed with the cell lysis buffer. Equivalent amounts of the sample lysate were then separated through sodium dodecyl sulfate polyacrylamide gel electrophoresis, which were later transferred onto the nitrocellulose membrane (Millipore) by electroblotting. Later, the membrane was blocked with 5% non-fat milk in the TBST buffer (containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.1% Tween 20) at 4 ℃ overnight, followed by incubation with specific primary antibodies for 2 h and the specific IgG HRP-conjugated secondary antibody for 1 h at room temperature. Details for the primary antibodies were as follows: Slug (1:2,000, catalog number # ab180714) and FoxC2 (1:2,000, catalog number #ab5060) were obtained from Abcam (Cambridge, MA, USA), β-actin (1:1,000, catalog number #bs-10021) was purchased from Bioworld (CA, USA). The resultant signals would then be visualized through enhanced chemiluminescence (ECL) (Pierce) on the Syngene G: BOX Chemi gel documentation system (Syngene, Cambridge, UK). The densitometric values would be normalized in each group, with β-actin being used as the internal reference.

Real-time quantitative PCR analysis

Cells in each group were harvested at 48 h after transfection, and the total RNA would then be isolated using the TRIzol kit (Invitrogen, CA, USA) in accordance with the manufacturer's instructions. 1 μg total RNA was used to synthesize cDNA using a PrimeScript reagent Kit with DNAEraser (Takara Bio, Kyoto, Japan), followed by RT-PCR analysis using the SYBR green Master Mix (Takara Bio, Kyoto, Japan). The following primer pairs were obtained from Invitrogen (NY, USA), while GAPDH served as the internal reference.

GAPDH: 5'-GAAGAGTCAAGAGCAGTCGTCAAG A-3' (forward), 5'-GTAAGACCGAACCACAGTCAAGAG AG-3' (reverse); Slug: 5'-GAGCCTTCAAGAGAAAAAAG GAATT-3' (forward), 5'-GTAGTCAAGAGTCTTTTTT CCTTAA-3' (reverse); FoxC2: 5'-GAGCCTTCAAGAGC CAGGTGGAATT-3' (forward), 5'-GTTCCGTCTTCTC AAGAGACGAATA-3' (reverse).

CCK-8 colorimetry assay

Ten μL CCK-8 was added into each well at 24, 48, 72 and 96 h, respectively, and further cultured for 4 h, and the optical density value in each well was measured using a microplate reader. Subsequently, the growth curve was plotted, and the average of 4 wells in each group was used to calculate the cell proliferation capacity. Each experiment was repeated for three times.

Transwell chamber migration assay

Cells in each group were digested with trypsin and washed with the serum-free culture medium. Cell suspension was prepared, and 200 μL was evenly added into the upper Transwell chamber (Beyotime Biotechnology, Haimen, China), while 600 μL complete medium was added to the lower Transwell chamber for 48 h. Then, the chambers were taken out, washed with PBS, fixed with 4% paraformaldehyde for 20 min, and stained with 1% crystal violet staining solution for 10 min. Subsequently, cells on the basilar membrane surface were wiped using the cotton swab, and the number of cells penetrating the membrane was counted under microscope. Each experiment was repeated for three times.

Transwell invasion assay

Before Transwell invasion assay, cells were deprived of serum for 24 h, resuspended (5×10^4) with 200 μL serum-free medium after trypsin digestion, and added onto the Matrigel surface in the upper Transwell chamber, while 600 μL complete medium was added to the lower Transwell chamber as the chemotaxin. The number of cells penetrating the Matrigel was counted under microscope. Each experiment was repeated for three times.

ELISA assay

The concentrations of MMP-2 and MMP-9 were further evaluated in both cell lines. Cell culture supernatants were collected at 48 h after transfection. MMP-2 and MMP-9 concentrations were measured using human MMP-2 ELISA Kit and human MMP-9 ELISA Kit (Invitrogen

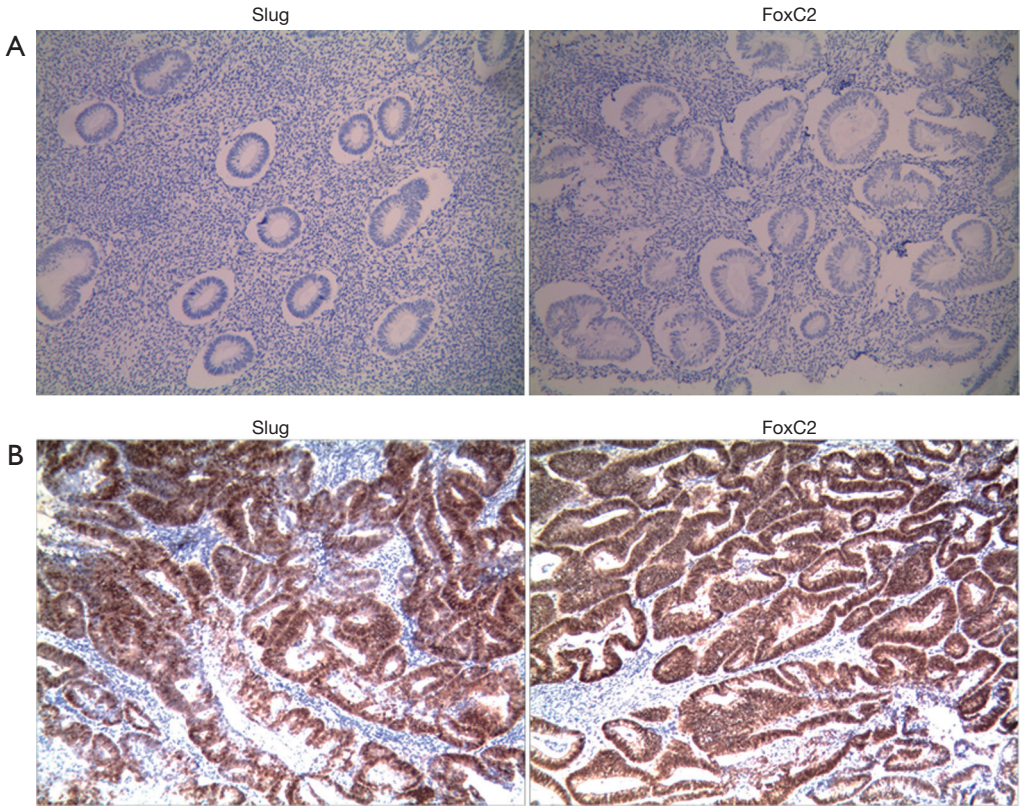

Figure 1 Expression of Slug and FoxC2 in the normal control (A) and endometrial carcinoma (B) tissues was analyzed using immunohistochemical staining (×100).

Corporation, Carlsbad, United States) according to the manufacturer's instructions. The optical density (OD) value at 450 nm was record using a microplate reader.

Statistical analysis

Statistical analyses were performed using SPSS 18 for Windows (SPSS Inc., Chicago, IL, USA). Data are given as means ± SD and One-way analysis of variance followed by post-hoc Bonferroni analysis of variance. The two groups were compared with Student's t-test. Each experiment was repeated for three times. $P<0.05$ was taken as a significant difference between groups.

Results

Slug and FoxC2 were highly expressed in endometrial carcinoma tissues

As shown in *Figure 1*, immunohistochemical results indicated positive expression in the nuclei of endometrial carcinoma tissues. Compared with normal endometrial tissues, the Slug and FoxC2 expression levels in endometrial carcinoma tissues were remarkably increased. The positive rate of Slug and FoxC2 in endometrial carcinoma was 61.3% (76/124) and 71.0% (88/124), and the difference was statistically significant compared with that in normal endometrial tissues (*Table 1*).

Relationships of Slug and FoxC2 expression with the clinicopathological factors of endometrial carcinoma

There was no relationship between the Slug and FoxC2 expression and age in each group ($P>0.05$). Slug expression in endometrial carcinoma was notably correlated with the histological grade, muscular layer infiltration and lymph node metastasis ($P<0.05$), but it was not markedly correlated with The International Federation of Gynecology and Obstetrics (FIGO) stage. FoxC2 expression was distinctly related to the FIGO stage and lymph node metastasis ($P<0.05$), but it was not correlated with histological grade

Table 1 Expression of Slug, FoxC2 in distinct tissue types

Histological type	Cases	Slug			Fox		
		(–)	(+)	P value	(–)	(+)	P value
Normal endometrial tissues	35	27	8	<0.01	31	4	<0.01
Endometrial carcinoma tissues	124	48	76		36	88	

P<0.01 *vs.* normal and atypical hyperplastic group.

Table 2 The association of Slug, FoxC2 expression with clinicopathological features in endometrial carcinoma

Factors	Cases	Slug			FoxC2		
		(–)	(+)	P	(–)	(+)	P
Age							
<50	29	10	19		16	13	
≥50	95	38	57	0.593	50	45	0.810
Histological grade							
G_1	57	29	28		33	24	
G_2	41	14	27		20	21	
G_3	26	5	21	0.018*	13	13	0.627
FIGO stage							
I + II	101	42	59		59	42	
III + IV	23	6	17	0.168	7	16	0.015#
Muscular layer infiltration							
≤1/2	84	39	45		49	35	
>1/2	40	9	31	0.011&	17	23	0.099
Lymph node metastasis							
No	93	41	52		55	38	
Yes	31	7	24	0.033$	11	20	0.022$

*, P<0.05 *vs.* G1 and G2; #, P<0.05 *vs.* I + II stage; &, P<0.05 *vs.* invasion ≤1/2 group; $, P<0.05 *vs.* absent group.

and muscular layer infiltration (*Table 2*).

Slug and FoxC2 protein expression were detected by Western blotting and RT-PCR

Ishikawa and RL-952 cells were first observed at 48h after shRNAs transfection (*Figure 2*). The fluorescence images indicated that the cells were successfully transfected with the shRNAs. Western blotting and RT-PCR results also indicated that, compared with control group, Slug and FoxC2 protein and mRNA levels in Ishikawa (*Figure 3*) and RL-952 (*Figure 4*) cells were remarkably reduced after Slug-shRNA and FoxC2-shRNA interference (P<0.01). Non-specific shRNA group showed no significant difference compared with those in blank control group (P>0.05).

Slug-shRNA and FoxC2-shRNA inhibited endometrial carcinoma cell proliferation

As shown in *Figure 3D* and *Figure 4D*, the proliferation rates of both cell lines in the control group were markedly higher than those in shRNAs treatment groups. Independent sample *t*-test results suggested that, the differences in the proliferation capacity of cells in expression vector treatment

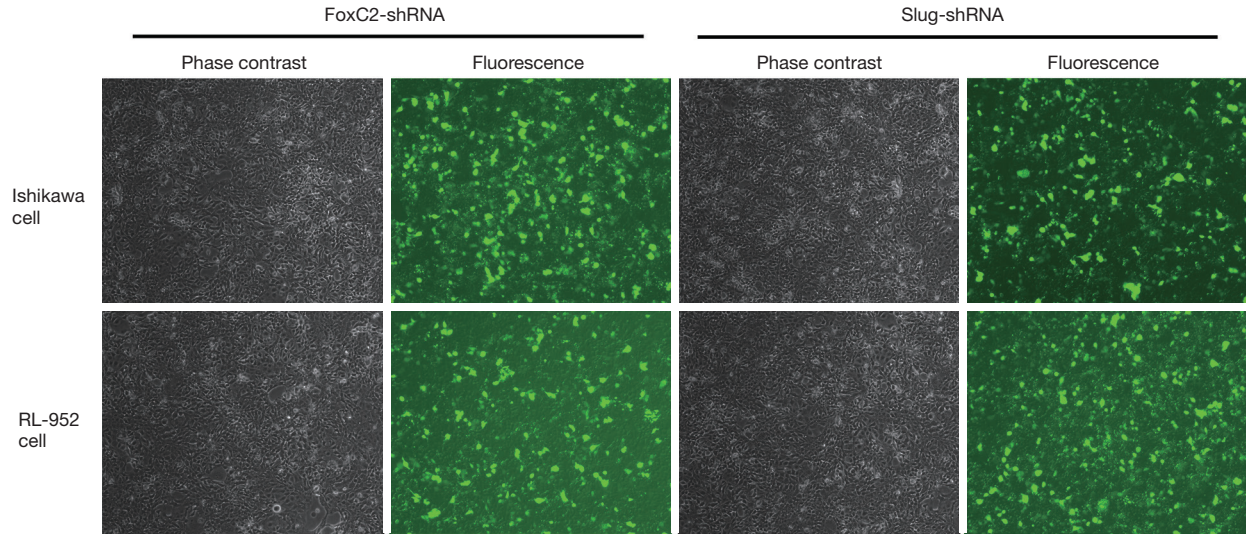

Figure 2 Ishikawa and RL-952 cells were observed at 48 h after transfection with Slug-shRNA and FoxC2-shRNA (×100).

groups were statistically significant compared with that in the other two groups at days 2, 3, 4, 5, 6 and 7 of growth (P<0.05). CCK-8 assay suggested that, the proliferation capacity of cells in shRNAs treatment groups was apparently lower than that in the control group.

Slug-shRNA and FoxC2-shRNA inhibited endometrial carcinoma cell migration and invasion

The migration and invasion abilities in Ishikawa and RL-952 cells were then evaluated. The numbers of Ishikawa cells penetrating the filter membrane into the lower chamber in Slug-shRNA and FoxC2-shRNA groups were 174.58±8.11 and 165.33±7.65, respectively. The numbers of cells penetrating Matrigel into the lower chamber in Slug-shRNA and FoxC2-shRNA groups were 94.02±6.57 and 84.56±8.36, respectively. While those in the control group and blank control group were 261.76±10.11 in migration assay and 175.02±9.65 in invasion assay (*Figure 5*). The results in RL-952 cell lines were similar with the Ishikawa cells. The numbers of cells penetrating the filter membrane into the lower chamber in Slug-shRNA and FoxC2-shRNA groups were 148.72±6.54 and 136.48±5.22, respectively. The numbers of cells penetrating Matrigel into the lower chamber in Slug-shRNA and FoxC2-shRNA groups were 76.42±5.63 and 77.82±7.32, respectively. While those in the control group 198.52±9.72 in migration assay and 178.63±6.38 in invasion assay (*Figure 6*). Compared with the control group, the numbers of cells entering the lower chamber in Slug-shRNA and FoxC2-shRNA groups in both cell lines were significantly reduced (P<0.05).

Slug-shRNA and FoxC2-shRNA reduced MMP-2 and MMP-9 in endometrial carcinoma cells

Subsequently, we compared the MMP-2 and MMP-9 of the cell culture supernatant in each group of both cell lines by ELISA assay. As shown in *Figure 7*, the expressions of MMP-2 and MMP-9 after shRNA-Slug and shRNA-FoxC2 transfection were significantly reduced. This is consistent with the results of our previous migration and invasion results.

shRNA-Slug and shRNA-FoxC2 inhibited EMT in endometrial carcinoma

To gain insights into the mechanism underlying the Slug and FoxC2 inhibiting endometrial carcinoma migration and invasion, we evaluated the protein level of EMT related protein including E-cadherin, N-cadherin and Vimentin by Western blotting. As shown in *Figure 8*, after transfection of shRNA-Slug and shRNA-FoxC2 in Ishiyama and RL-952 endometrial carcinoma cells, the expression of E-cadherin increased while the expression of N-cadherin and Vimentin decreased. These results indicated that EMT capability is suppressed by shRNA-Slug and shRNA-FoxC2.

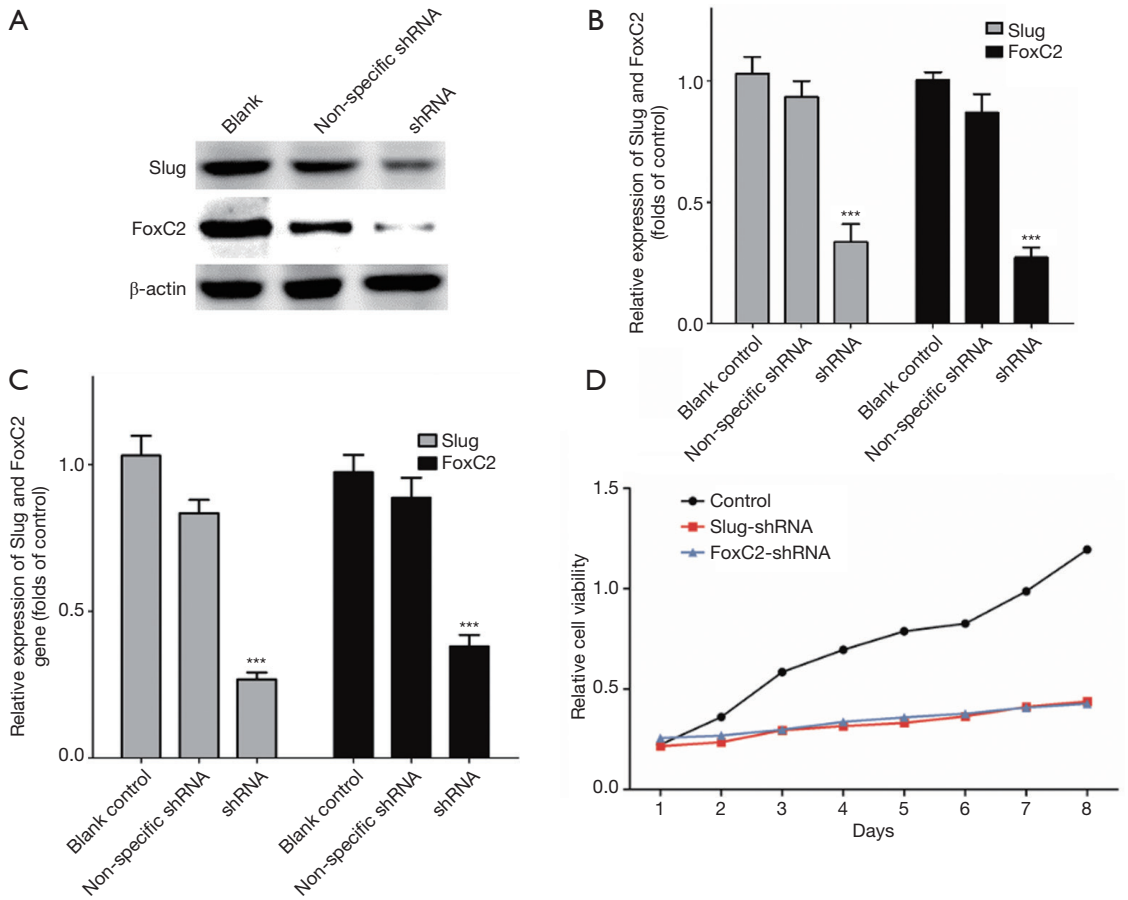

Figure 3 Slug-shRNA and FoxC2-shRNA reduced Ishikawa cells proliferation. (A) The protein expression in each group was detected using western-blot. Representative images for Slug and FoxC2 were shown; (B) quantification of Slug and FoxC2 expression; (C) the Slug and FoxC2 gene in each group was detected by RT-PCR; (D) Ishikawa cell viability was detected on the on the 1–8 days after transfection using CCK-8 method. Data represent the mean ± SD (n=3). ***, P<0.001 versus control group.

Discussion

In the current study, we found that Slug and FoxC2 expressions in endometrial carcinoma tissues were remarkably increased. In addition, Slug and FoxC2 silences by shRNA significantly inhibited endometrial carcinoma proliferation and metastasis, which may be related to the downregulation of MMPs protein expression and inhibition of EMT.

As a member of the Zinc finger transcription factor family members, Slug is mainly involved in neoplasm malignant phenotype regulation, and could also promote EMT, which is closely related to tumor cell migration and invasion (2,5). Recent researches have indicated that, Slug antisense could prevent EMT, indicating that, the Slug gene could act a treatment target for tumor invasion and metastasis (6-8).

FoxC2 is also referred to as mesenchyme forkhead 1 (MFH1), which belongs to the forkhead transcription factor family member and is encoded by genes located at human chromosome 16q24.1 (9,10). It has been highlighted that FoxC2 plays a central role in multiple signaling pathways, which are closely related to EMT and could further promote angiogenesis in tumor development (11,12).

In our current study, we found that the positive expression rates of Slug and FoxC2 proteins in endometrial carcinoma of 68.75% and 59.34%, respectively, which were remarkably elevated compared with that in normal endometrial tissue group (25.00%). Besides, the Slug and FoxC2 expressions were evidently correlated with the endometrial carcinoma development and poor prognosis

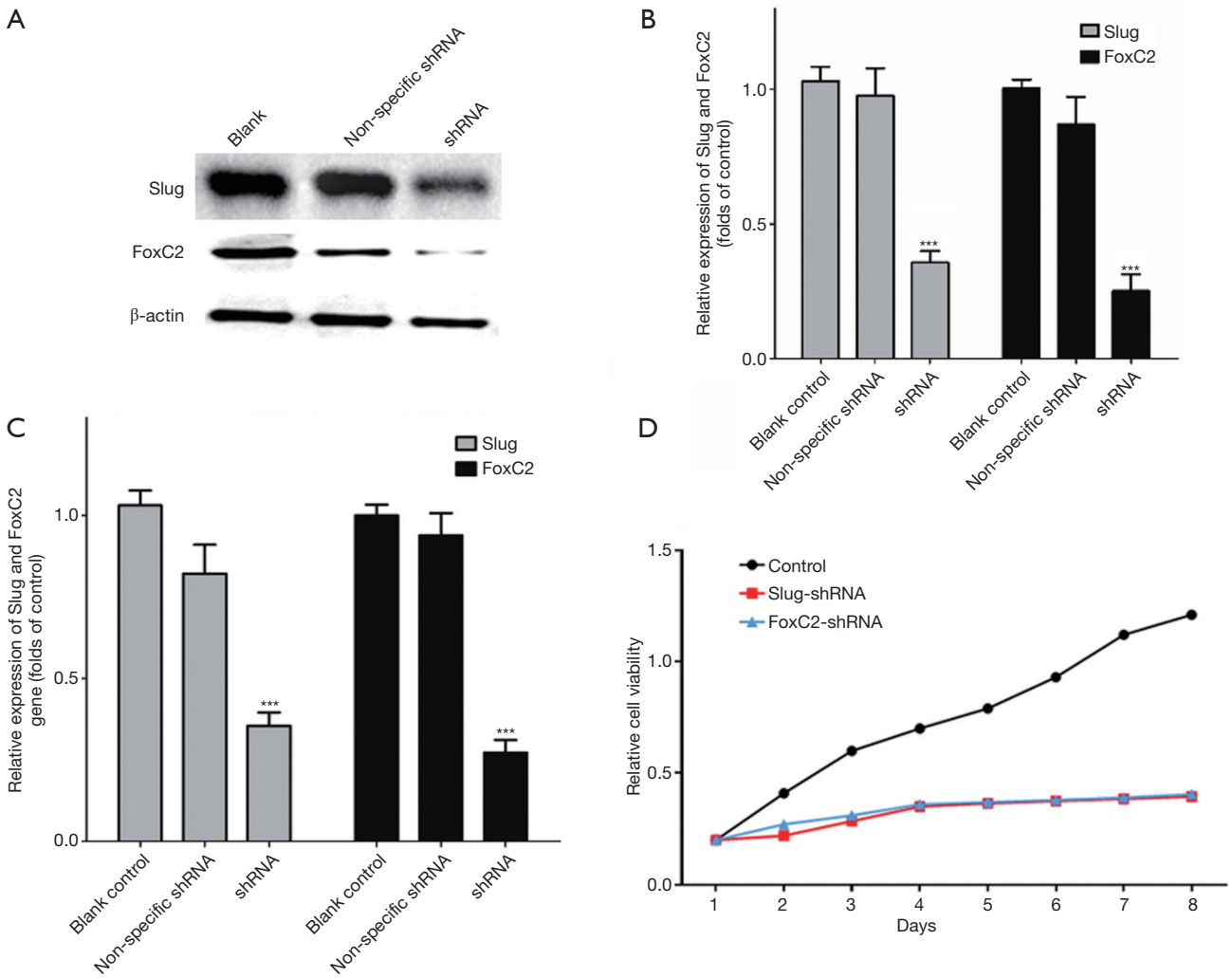

Figure 4 Slug-shRNA and FoxC2-shRNA reduced RL-952cells proliferation. (A) The protein expression in each group was detected using western-blot. Representative images for Slug and FoxC2 were shown; (B) quantification of Slug and FoxC2 expression; (C) the Slug and FoxC2 gene in each group was detected by RT-PCR; (D) RL-952 cell viability was detected on the on the 1-8 day after transfection using CCK-8 method. Data represent the mean ± SD (n=3). ***, P<0.001 versus control group.

(P<0.05). This suggests that Slug and FoxC2 may play a crucial role in endometrial carcinoma metastasis and Slug and FoxC2 could be used as an indicator for the prognosis of endometrial carcinoma.

In order to verify the Slug and FoxC2 roles in endometrial carcinoma, Ishikawa and RL-962 endometrial carcinoma cells were transfected with shRNA-Slug and shRNA-FoxC2 to silence the expression of Slug and FoxC2 respectively, which were confirmed by the fluorescence microscopy, Western blotting, and RT-PCR. Cell proliferation, invasion and migration were significantly inhibited by shRNA-Slug and shRNA-FoxC2 in both cell lines. The matrix metalloproteinases (MMP) family is a class of Zn^{2+} and Ca^{2+} -dependent endopeptidases that play an important role in multiple pathological processes such as inflammation, tumors, and cardiovascular diseases. It has found that MMP-2 and MMP-9 can promote tumor neovascularization *in vivo*, and upregulation of MMP-2 and MMP-9 in stromal cells could significantly increase the tumor cell metastasis (13). In the current study, our results also highlighted that the expressions of MMP-2 and MMP-9 in both cell lines after shRNA-Slug and shRNA-FoxC2 transfection was significantly decreased compared with the control group.

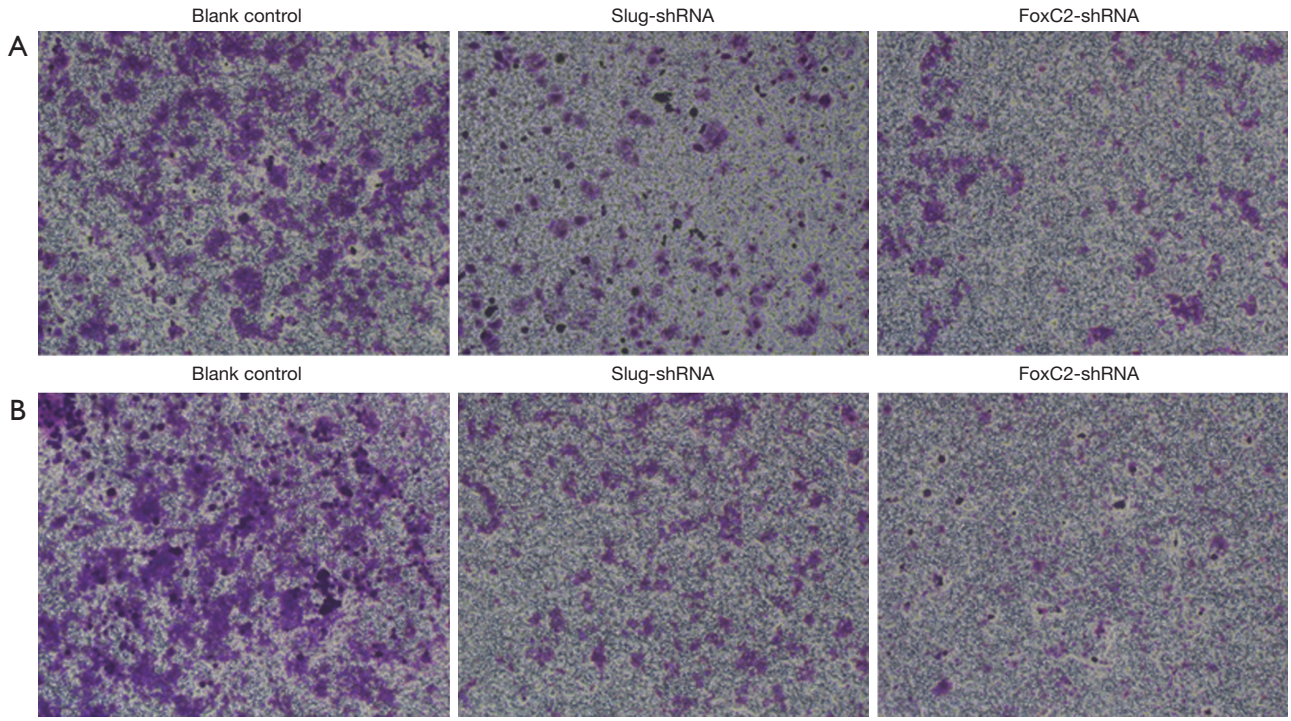

Figure 5 *In vitro* analysis of migration capacities (A) and invasion capacities (B) of Ishikawa cells after transfected with Slug-shRNA and FoxC2-shRNA (×100).

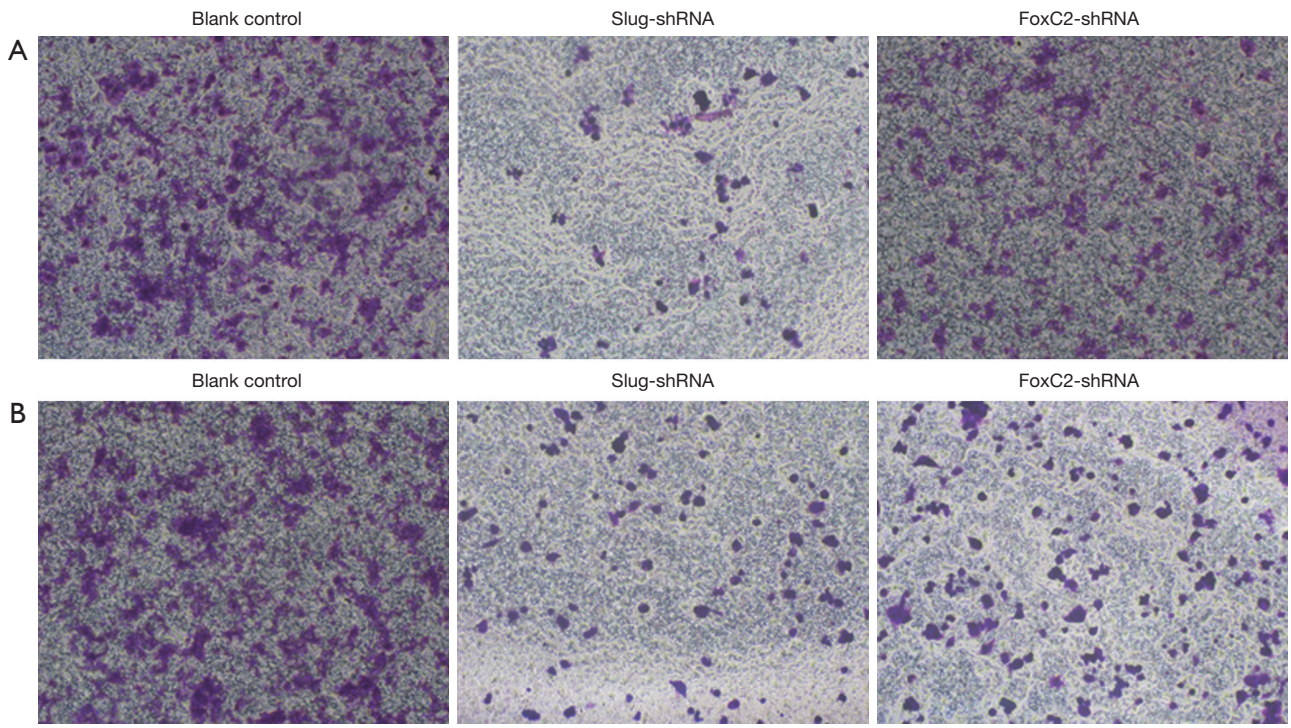

Figure 6 *In vitro* analysis of migration capacities (A) and invasion capacities (B) of RL-952 cells after transfected with Slug-shRNA and FoxC2-shRNA (×100).

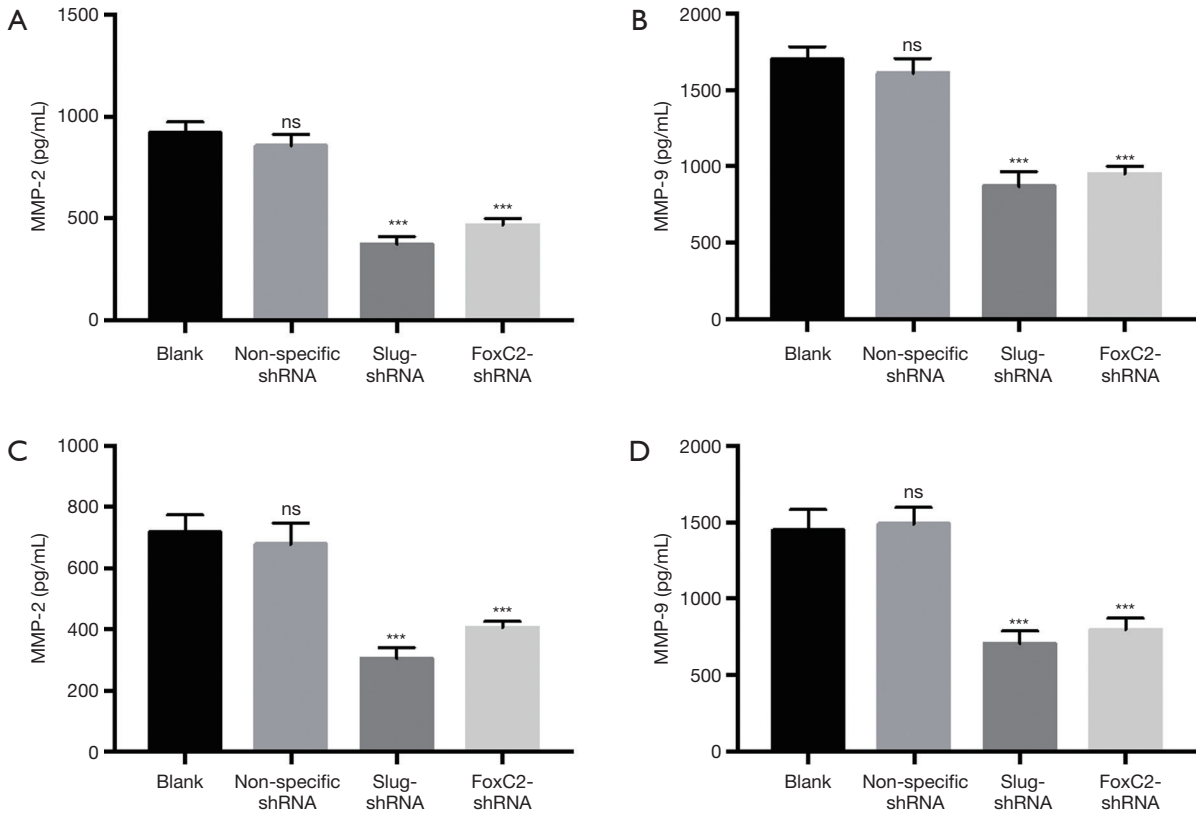

Figure 7 MMP-2 and MMP-9 concentrations in Ishikawa cells (A) and RL-952 cells (B) were detected using Elisa assay. Data represent the mean ± SD (n=3). ***, P<0.001 versus control group; ns, no statistical significance.

Infiltration and metastasis are also the main causes of treatment failure and poor clinical prognosis of endometrial carcinoma. It has been reported that metastasizes of endometrial carcinoma related to by EMT was induced by Slug and FoxC2 (14-19). EMT was an originally physiological process that occurred during the development of mammalian embryos, and recent research showed that EMT played an important role in the metastasis of tumors. Epithelial-derived tumor cells can be transformed into tumor cells with stronger migration ability and the enhanced invasions could also be promoted by interstitial cells. EMT could be activated by both intracellular signaling pathways and integrin signaling pathways, such as Slug, Snail, Twist and FoxC2 (20-25). Then the expressions EMT markers (such as Slug, Snail, and FoxC2) in tumor cells will be significantly increased, which will enhance cell invasiveness and lead to transformation into tumor cells (11,26,27), and the migration and resistance to apoptosis of the tumor epithelial cells will be enhanced (28-30). As EMT markers, E-cadherin, N-cadherin and Vimentin were analyzed in each group in both cell lines. We found that, after transfection of shRNA-Slug and shRNA-FoxC2, the expression of E-cadherin was markedly increased while the N-cadherin and Vimentin expression was significantly decreased, which was in consistence with previous studies (5,31), indicating the suppression of EMT by shRNA-Slug and shRNA-FoxC2.

Conclusions

In summary, our current study found that, compared with normal endometrial tissues, the Slug and FoxC2 expression levels in endometrial carcinoma tissues were remarkably increased. Interfering with Slug and FoxC2 through shRNAs can effectively suppress the *in vitro* proliferation, invasion, and migration of Ishikawa and RL-952cells, which may be related to the downregulation of MMPs protein and inhibition of EMT. Further studies will more focus on the detailed mechanism of the regulation of EMT in endometrial carcinoma by Slug and FoxC2.

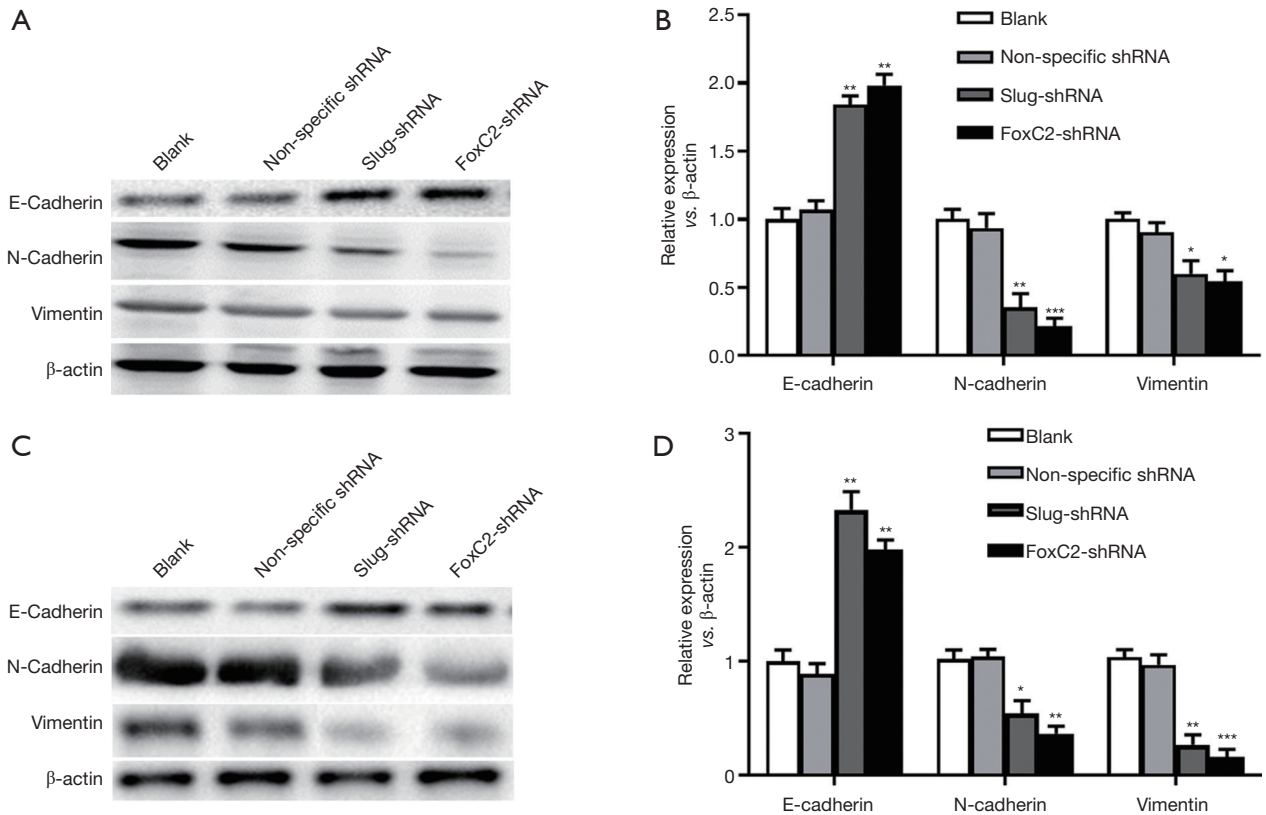

Figure 8 The expression of E-cadherin, N-cadherin and Vimentin in Ishikawa cells (A) and RL-952 cells (B) were detected by Western blotting. The representative images were shown and quantifications were performed. Data represent the mean ± SD (n=3). *P<0.05, **P<0.01, ***P<0.001 versus control group.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr-20-809). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by institutional ethics board of The First Affiliated Hospital of Bengbu Medical College (No. BBMEC-2018-10) and informed consent was taken from all the patients.

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