Upstream transcription factor 1 prompts malignancies of cervical cancer primarily by transcriptionally activating p65 expression

WEN WANG¹, SHUJUAN YAO¹, HONGJING JIANG¹, JING DONG¹, XIUJUAN CUI¹, XIANGYU TIAN², YANYAN GUO³ and SHIQIAN ZHANG⁴

Departments of ¹Obstetrics and Gynecology, and ²Medical Imaging, Tengzhou Central People’s Hospital, Tengzhou, Shandong 277500; ³Department of Obstetrics and Gynecology, Shandong Police Hospital, Jinan, Shandong 250001; ⁴Department of Obstetrics and Gynecology, Qilu Hospital, Shandong University, Jinan, Shandong 250012, P.R. China

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Abstract. Cervical cancer is the third-most common cause of female cancer-related mortality worldwide. In cervical cancer, aberrant activation of nuclear factor (NF)-κB signaling is widely reported. However, the transcriptional regulation of NF-κB signaling remains unclear. The present study aimed to explore the underlying mechanism in which NF-κB signaling was activated in cervical cancer cells. Initially, the expression of p65 was demonstrated to be markedly enhanced in grade II, III or IV cervical cancer tissues compared with that of normal cervical tissues, indicating that p65 expression was correlated with tumor grade. In HeLa and CaSki cells, overexpression of p65 markedly enhanced cervical cancer cell invasion and migration. Further experiments demonstrated that p65 overexpression significantly increased the phosphorylation levels of protein kinase B (AKT) and p38. Dual luciferase reporter and chromatin immunoprecipitation assays demonstrated that USF1 was able to bind the promoter region of p65, thereby enhancing the transcriptional activation of p65. Notably, when p65 was silenced, the phosphorylation levels of AKT and p38 were suppressed even in cells transfected with adenovirus vectors expressing upstream transcription factor 1 (USF1). These data indicated that USF1 prompted cervical cancer progression primarily by transcriptionally activating p65. In conclusion, the present study demonstrated that USF1 was able to activate the transcription of p65, thereby enhancing the malignancy of cervical cancer cells.

Introduction

Cervical cancer is the third most common cause of female cancer-related mortality and increased at a mean annual rate of 0.6% between 1980 and 2010 (1,2). At present, typical therapies for cervical cancer include surgery, radiotherapy and/or chemotherapy, alone or in combination (3,4). However, the recurrence and metastasis of cervical carcinoma remain problematic in clinical practice (5,6). Therefore, it is important to explore the underlying mechanisms that regulate the migration and invasion of cervical cancer cells.

Nuclear factor (NF)-κB induces a variety of biological processes via transcriptional gene control of key components in different signaling pathways (7,8). The complexes of NF-κB include five different subunits, namely RelA/p65, RelB, c-Rel, p50 and p52, which are able to form distinct homodimers and heterodimers (9,10). Classical NF-κB is a heterodimer that consists of the DNA binding subunit p50 and the transactivation subunit RelA/p65 (11). In the normal status, NF-κB remains in the cytoplasm and translocates into the nucleus following exposure to the stimuli, including inflammatory responses and hypoxia, thereby enhancing abnormal cell proliferation and survival (12). In cervical cancer, aberrant activation of NF-κB signaling is widely reported (13,14). However, the transcriptional control of NF-κB signaling remains unclear.

As a member of the basic helix-loop-helix leucine zipper (bHLH-LZ) family, the upstream transcription factor 1 (USF1) is widely associated with the transcription of many genes (15,16). Through binding the E-box motifs in the promoter region, USF1 is able to activate the transcription of target genes in the form of a homodimer or a heterodimer (USF1/2) (15). For instance, various genes associated with lipid and glucose metabolism have been reported to be modulated by USF1 (15-17). To the best of our knowledge, the expression and potential functional role of USF1 in cervical cancer has not been reported previously.

In the present study, the expression of USF1 was explored in cervical cancer. It was demonstrated that USF1 expression was markedly enhanced in grade II, III, and IV cervical cancer tissues compared with that of normal cervical tissues. Further experiments indicated that two E-box motifs were located at the promoter region of RelA/p65. A chromatin...
immunoprecipitation (ChIP) assay demonstrated that USF1 was able to activate the transcription of RelA/p65, thereby enhancing the malignant invasion and migration phenotype of cervical cancer cells.

Materials and methods

Ethics statement. Primary human cervical cancer patient specimens were obtained from patients and informed consent was obtained under protocols approved by Review Boards of Shandong University (Jinan, China). Biometric samples from patients with cervical cancer were frozen in liquid nitrogen and stored at -80˚C. The present study was approved by the Ethics Committee of Tengzhou Central People's Hospital (Tengzhou, China). A total of 10 cervical cancer tissues (age, 52.3±12.5) and 5 normal adjacent para-cervical cancer tissues (age, 48.7±11.6, female) were collected from patients at the Tengzhou Central People's Hospital (Tengzhou, China) between November 2012 and December 2012. The tissues were analysed to determine the clinical staging and clinicopathological characteristics of each case of cervical cancer. Diagnoses were made in accordance with the altered International Federation of Gynecology and Obstetrics staging system (18). There was 1 patient at Stage I, 3 patients at Stage II, 3 patients at Stage III and 3 patients at stage IV. All patients enrolled in the present study were diagnosed with gynaecological tumours. The inclusion criteria for the present study were as follows: i) Pathological diagnosis confirmed by a minimum of 2 pathologists; ii) patient received a thorough pre-treatment evaluation, including a detailed medical history, physical examination, whole blood cell count, liver and kidney function tests, imaging examination (chest X-ray, color Doppler ultrasound, computed tomography, magnetic resonance imaging, and positron emission tomography), electrocardiogram, comprehensive assessment of electronic colposcopy for vulva, vagina, cervix and cystoscopy. Colonoscopy was also included in the presence of a clinical indication; iii) the clinical stage of cervical cancer was determined by 3 gynecologic-oncologists; iv) patients had complete records of clinicopathological and follow-up examinations and had also signed the informed consent document. Patients were excluded if they had received preoperative chemotherapy or radiotherapy, or if they could not be contacted during follow-ups. All participants in the present study provided their written informed consent regarding the use of their clinical material in the research described. Once the samples were obtained under protocols approved by Review Boards of Shandong University (Jinan, China). In brief, HeLa cells were seeded at a density of 10^5 cells/well for 24 h. Ad-USF1, Ad-USF1i or Ad-NC was subsequently transfected into HeLa cells for 48 h at 37˚C. The RNA was collected after the above adenovirus vectors were transfected into HeLa cells for 48 h and used for further analysis.

ChIP. ChIP assay was performed using the Chromatin Immunoprecipitation Assay kit (EMD Millipore, Billerica, MA, USA). Briefly, the nucleic DNA was extracted from cells and sonicated into 200-1,000 bp. Precleared chromatin was immunoprecipitated with anti-USF1 (1:100; ab180717) and immunoglobulin G (1:100; ab172730) (both Abcam, Cambridge, UK) antibodies according to the manufacturer's protocol. Immunocomplexes were added into 50 µl protein A/G-Sepharose beads and purified with Qiaquick (Qiagen GmbH, Hilden, Germany) polymerase chain reaction (PCR) purification columns. The DNA of HeLa and CaSki cells was quantified on a NanoDrop™ ND3300 (Thermo Fisher Scientific, Inc., Waltham, MA) using a Quant-iT Picogreen dsDNA Assay kit (Invitrogen; Thermo Fisher Scientific, Inc.) and equal quantities of DNA were used as the template. The precipitated DNA was amplified with p65-specific primers. The primers specific to the USF1 binding sites on the p65 promoter were as follows: Forward, 5'-GTACCAGAGGT GATTCTGC-3'; and reverse 5'-AGGCTACTCTGAGTGGCC TT-3'. Quantitative PCR was performed using SYBR-Green PCR Master mix (Roche Diagnostics, Basel, Switzerland) on an Applied Biosystems ViIA 7 Real-time PCR system (Thermo Fisher Scientific, Inc.). The final reaction volume was 10 µl and contained 5 µl SYBR-Green PCR Master mix (2X), 0.5 ml forward and 0.5 µl reverse primers (10 mM), 2 ml cDNA and 2 µl double-distilled water. The procedure used for qPCR was as follows: 95˚C for 10 min followed by 50 cycles of 95˚C for 10 sec, 55˚C for 10 sec, 72˚C for 5 sec; 99˚C for 1 sec; 59˚C for 15 sec; 95˚C for 1 sec; then cooling to 40˚C. Comparison of input, USF1 pulldowns were reported as the average according to the manufacturer's protocol (EMD Millipore).

RNA extraction and reverse transcription-quantitative (RT-q) PCR. The total RNA from primary human cervical cancer tissues was isolated using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. The total RNA was reverse transcribed into cDNA using a TaqMan RNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). qPCR was performed using SYBR-Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) in a Bio-Rad iCycler® real-time PCR detection system as described above. GAPDH was used as the internal control. The primers used were as follows: P65 forward, 5'-ATCCCCATCCTCCAGCTTCT-3' and reverse 5'-AGGACCTCTGTAGGGCAG-3'; GAPDH forward 5'-GAGAAGGCCGCTGGGCTCATT-3' and reverse 5'-AGTGATGGCATGAGCTTGG-3'.

Protein extraction and western blot analysis. Proteins samples were extracted from primary human cervical cancer tissues or normal adjacent para-cervical cancer tissues and cultured HeLa and CaSki cells in radioimmunoprecipitation assay
buffer (1% TritonX-100, 15 mmol/l NaCl, 5 mmol/l EDTA and 10 mmol/l Tris-HCl; pH 7.0; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) supplemented with a protease and phosphatase inhibitor cocktail (Sigma-Aldrich; Merck KgaA, Darmstadt, Germany.) A bicinechonic protein assay kit (Pierce; Thermo Fisher Scientific, Inc.) was used to determine the protein concentration. Equal quantities of protein (15 µg) were separated by 10% SDS-PAGE and electrophoretically transferred to a PVDF membrane. Following blocking with 8% milk in PBS with Tween-20 (pH 7.5) for 2 h at room temperature, membranes were incubated with the following primary antibodies at 4°C overnight: Anti-USF1 (cat. no. ab180717; 1:1,000, Abcam), anti-p-p65 (cat. no. ab86299; 1:1,000; Abcam), anti-p65 (cat. no. ab180717; 1:1,000; Abcam), anti-phosphorylated (p)-protein kinase B (Akt; cat. no. 13038; 1:1,000), anti-AKT (cat. no. 5084; 1:1,000), anti-p-p38 (cat. no. 8632; 1:1,000.), anti-p38 (cat. no. 8690; 1:1,000) and anti-GAPDH (5174; 1:1,000) (all Cell Signaling Technology, Inc., Danvers, MA, USA). Following several washes with TBST the membranes were incubated with horserasish-peroxidase (HRP)-conjugated goat anti-rabbit (cat. no. ZB-2306; 1:5,000; OriGene Technologies, Inc., Beijing, China) for 2 h at room temperature and then washed. Immunodetection was performed using the Immobilon Western Chemiluminescent HRP Substrate (cat. no. WBKLS0500; EMD Millipore) enhanced chemiluminescence detection system according to the manufacturer's protocol. The house-keeping gene GAPDH was used as the internal control. ImageJ 1.43b software (National Institutes of Health, Bethesda, MD, USA) was used for density analysis.

**Invasion and migration assays.** HeLa and CaSki cells were seeded in DMEM culture in the top chamber of each Transwell insert (BD Biosciences, San Jose, CA, USA) at 1.0x10^5 cells/well with 8.0-mm pores for the migration assay. For the invasion assay, 2.0x10^5 cells were cultured in DMEM culture in the upper chamber of a Transwell insert (BD Biosciences) at 37°C for 24 h that was pre-coated with 0.2% Matrigel (Oncient Pharmaceuticals Corporation, Waltham, MA, USA) at 37°C. As a chemoattractant, 10% fetal bovine serum was added to the DMEM culture medium in the lower chamber. Then, Ad-p65 or Ad-NC/Ad-USF1 or Ad-NC was placed into the lower chamber at 37°C for 24 h. Following incubation for 24 h at 37°C, remaining cells in the upper chamber were removed using cotton swabs, and those which had migrated or invaded through the membrane were stained with a dye solution containing 20% methanol and 0.1% crystal violet at 37°C for 15 min. Cells were subsequently imaged under a light microscope (magnification, x40; Olympus Corporation, Tokyo, Japan) and 10 individual fields were randomly chosen and counted per insert. The results are presented as the mean of three separate experiments.

**Promoter reporter analysis.** The promoter region of p65 was amplified from the genomic DNA of HeLa cells by PCR. The pGL3 Basic vector (Promega Corporation, Madison, WI, USA) and the amplified fragments were digested with XhoI/KpnI (New England BioLabs, Inc., Ipswich, MA, USA) and purified by 2% agarose gel electrophoresis. The reaction mix included 10X buffer (including Mg^2+), 5 µl; 2.5 mM dNTP, 2 µl; 10 µM forward primer, 1 µl; 10 µM reverse primer, 1 µl; template, 2 µl; Taq DNA polymerase, 0.5 µl (Takara Bio, Inc., Otsu, Japan); dDNA2O 38.5 µl (Takara Bio, Inc.) The procedure included 94°C for 5 min; 30 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min; and 72°C for 5 min. The primer sequences used for the PCR reaction were as follows: p65 forward, CAAGAGCCCCATTAGCTC; and p65 reverse, CAAGAGCCCCATTAGCTC. The digested fragment was then inserted into the pGL3 vector upstream of the SV40 promoter. 293 (American Type Culture Collection) cells were co-transfected with the pGL3 plasmids and the PRL-TK vector (Promega Corporation) using the Vigofect Transfection Reagent (Vigorous Biotechnology Co., Ltd., Beijing, China). The cells were harvested and lysed 48 h post-transfection. The relative light units were determined using a Dual-luciferase Reporter Assay system (Promega Corporation) according to the manufacturer's protocols. Normalized luciferase data (firefly/Renilla) was compared with the empty pGL3-Basic vector.

**Immunohistochemistry.** Harvested primary human cervical cancer tissues or normal adjacent para-cervical cancer tissue samples were fixed in 4% phosphate-buffered formalin at room temperature for 20 min, embedded in paraffin and cut into 5-µm thick sections, followed by deparaffinization, rehydration in a descending series of alcohol and microwave-heating in sodium citrate buffer (Beijing Solarbio Science & Technology Co., Ltd.) at 100°C for 30 min for antigen retrieval. Sections were subsequently incubated with 0.3% hydrogen peroxide/phosphate-buffered saline for 30 min. The sections were incubated with a primary anti-p65 antibody (1:100) at a 1:50 dilution and 4°C overnight. Detection of the primary antibody was performed via incubation with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (ZDR-5036; OriGene Technologies, Inc.) for 1 h at room temperature and visualized with a 3,3’-Diaminobenzidine substrate. Stained cells were counted in 5 random fields using an Olympus CK40 light microscope (magnification, x40; Olympus Corporation).

**Transient transfection.** Initially 6x10^5 cells were equally seeded in 6-well plates with 2 ml DMEM medium containing serum and antibiotics as described above. Small interfering RNA targeting p65 (5’-CGATTCTTTAACCCTCAGAGT-3’) or negative control (5’TTCCTCAGACTGTCTAGT-3’) were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). Simultaneously, siRNA-p65 or -NC were mixed with HiPerFect transfection reagent (Qiagen GmbH, Hilden, Germany) and incubated at room temperature for 10 min. Each complex was subsequently transfected into two wells containing the HeLa cells for 48 h at 37°C at a final concentration of 10 nM in the presence or absence of Ad-USF1. To further evaluate whether USF1 exerts its role through p65, Ad-USF1 or Ad-NC was transfected into HeLa cells for 24 h. Then, si-p65 or si-NC was transfected into the above cells with Ad-USF1 or Ad-NC for another 48 h as described above. Following transfection for 48 h the cells were collected for further analysis.

**Statistical analysis.** Data were presented as the mean ± standard deviation from three independent experiments. Statistical
analysis was performed using Student's t-test for comparisons between two groups, and one-way analysis of variance followed by a post hoc Tukey's test were used for comparisons of two more groups, using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Overexpression of p65 in cervical cancer tissues. Initially, the expression of p65 was evaluated in cervical cancer tissues. Compared with normal cervical tissues, the expression of p65 was markedly higher in cervical cancer tissues (Fig. 1A). Furthermore, compared with normal tissues the expression of p65 was markedly enhanced in grade II, III or IV cervical cancer tissues, whereas less obvious changes were observed in grade I cervical cancer tissues (Fig. 1A), which suggests that p65 expression was correlated with tumor grade. Furthermore, the mRNA level of p65 was also significantly enhanced in cervical cancer tissues (grades II-IV) compared with that of control (Fig. 1B). No significant changes in the mRNA level of p65 were observed in the grade I cervical cancer tissues (data not shown).

p65 enhances cervical cancer cell invasion and migration. The role of p65 in cancer cell invasion and migration was explored. Ectopic expression of p65 was introduced in HeLa and CaSki cells using adenovirus vectors overexpressing p65 (Ad-p65) compared with that of controls (Ad-NC). It was demonstrated that transfection with Ad-p65 in HeLa and CaSki cells markedly increased cancer cell invasion and migration capacities (Fig. 2A). Furthermore, the downstream effectors, including AKT and p38 signaling, were also investigated. As shown in Fig. 2B, overexpression of p65 significantly enhanced the phosphorylation levels of AKT and p38 in HeLa cells, which suggests an oncogenic role of p65 in cervical cancer cells.

USF1 activates p65 expression. Whether USF1 was able to activate the transcription of p65 was also investigated. In multiple genes, an enhancer (E)-box lies within the promoter region to provide a binding site for members of the basic helix-loop-helix (bHLH) transcription factor family and promotes the transcription of the downstream gene (19). USF1 belongs to the eukaryotic evolutionary conserved basic-Helix-Loop-Helix-Leucine Zipper transcription factor family and demonstrates higher binding affinity for E-box elements (20). Notably, two E-box elements were identified in the promoter region of p65 (Fig. 3A). Subsequently the expression of USF1 in cervical cancer tissues was evaluated. Compared with normal cervical tissue, the expression of USF1 was markedly enhanced in the cervical cancer tissues (Fig. 3B). Subsequently, the promoter region of p65 was cloned into the pGL3 Basic reporter vector. A dual luciferase reporter assay demonstrated that inhibition of USF1 was able to significantly decrease the relative luciferase reporter activity of pGL3-p65 compared with blank vector (Fig. 3C). Furthermore, a ChIP assay demonstrated that the knockdown of USF1 expression in HeLa cells significantly reduced the interaction between USF1 and the p65 promoter (Fig. 3D), whereas the over-expression of USF1 significantly enhanced its interaction with p65 promoter (Fig. 3E). These data suggested that USF1 was able to bind the promoter region of p65.

Overexpression of USF1 enhances cervical cancer cell invasion and migration by transcriptionally activating p65. The functional role of USF1 on HeLa and CaSki cancer cell migration and invasion was evaluated. It was demonstrated that migration and invasion capacities were markedly increased in cervical cancer cells transfected with Ad-USF1 compared with that of Ad-NC (Fig. 4A). In addition, the protein level of p65 was significantly enhanced when USF1 was overexpressed in HeLa cells compared with controls (Fig. 4B). Furthermore, the expression of p65 was also significantly enhanced in HeLa cells overexpressed with USF1 (Fig. 4B). To further determine
Figure 2. p65 enhanced cell invasion and migration capacities. (A) Invasion and migration assays. (B) Western blot analysis of AKT and p38 activation when p65 was overexpressed. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.05 vs. Ad-NC. Ad-NC, control adenovirus; Ad-p65, adenovirus vectors overexpressing p65; p, phosphorylated; AKT, protein kinase B.

Figure 3. USF1 was able to activate the expression of p65. (A) Two E-boxes were identified in the promoter region of p65. (B) The expression of USF1 was markedly enhanced in cervical cancer tissues. (C) Dual luciferase reporter assay demonstrated that silencing USF1 was able to significantly reduce the relative luciferase reporter activity of pGL3-p65 compared with blank vector. (D) ChIP assay demonstrated that the knockdown of USF1 expression in HeLa cells reduced the interaction between USF1 and the p65 promoter. (E) ChIP assay demonstrated that the over-expression of USF1 enhanced its interaction with p65 promoter. Data are presented as the mean ± standard deviation of three independent experiments. ***P<0.001 vs. pGL3; **P<0.01 vs. Ad-NCi; *P<0.05 vs. Ad-NC. USF1, upstream transcription factor 1; ChIP, chromatin immunoprecipitation; TSS, transcription start site; RLU, relative light units; Ad -NCi, negative control adenovirus vectors for inhibiting gene expression; Ad-USF1i, adenovirus vectors for inhibiting USF1; Ad-NC, control adenovirus; Ad-USF1, adenovirus vectors overexpressing USF1.
whether USF1 exerts its role through p65, the expression of p65 was silenced in HeLa cells. As shown in Fig. 4C, when p65 was inhibited, the phosphorylation levels of AKT and p38 were significantly suppressed even in cells transfected with ad-USF1 (Fig. 4C). These data suggest that USF1 induced cervical cancer cell migration and invasion mainly via p65.

**Discussion**

Cervical cancer is regarded as the third-most common cause of cancer-related mortality among women worldwide (21). Recent studies have demonstrated that metastasis, rather than the original tumor, is the predominant cause of cancer-related mortality; therefore, it is of great importance to suppress cancer cell metastasis (22,23).

Abnormal activation of NF-κB serves a key role in modulating cancer-cell migration and invasion (24). In cervical cancer, constitutive NF-κB activation has been demonstrated to enhance tumor progression and is suggested to be correlated with poor prognosis (25). Typically, p65 forms a heterodimer with p50 and remains in the cytoplasm. Following exposure to stimuli including, hypoxia or inflammation the inhibitory subunit is degraded and the heterodimer of NF-κB is transported from the cytoplasm into the nucleus (26). Through
binding to the xB site in the promoter region of many target genes, NF-kB is associated with numerous target genes (27). It has been previously reported that suppression of NF-kB activity is able to inhibit cancer cell migration, invasion, angiogenesis, and metastasis (28,29). In the present study, it was demonstrated that p65 was upregulated in cervical cancer tissues compared with normal cervical tissues. In accordance with previous studies, it was demonstrated that overexpression of p65 markedly enhanced cancer cell migration and invasion. Notably, NF-kB is known to activate p38 mitogen-activated protein kinase (MAPK) and AKT, which subsequently regulate the expression of various genes associated with cancer cell invasion (30). In the present study, western blot analysis indicated that overexpression of p65 significantly enhanced the phosphorylation level of p38 MAPK and AKT, thereby prompting the migration and invasion process.

USF1 is a ubiquitous transcription factor of the bHLH-LZ family that is widely associated with lipid and glucose metabolism (31,32). However, whether it is associated with the progression of cervical cancer remains to be elucidated. In the present study, the expression of USF1 in cervical cancer tissues was explored and it was demonstrated to be significantly upregulated compared with normal cervical tissues. Notably, two conserved E-box elements were identified in the promoter region of p65. ChIP assay and luciferase reporter assay demonstrated that USF1 was able to bind the promoter region of p65 and significantly activate the transcription of p65 in cervical cancer cells. Further experiments demonstrated that USF1 was also able to enhance HeLa cell migration through AKT and p38 activation. Notably, it was determined that USF1-mediated activation of AKT/p38 signaling was partially abolished by p65 silencing, which suggests that USF1 enhanced the malignancies of cervical cancer cells primarily by transcriptionally activating p65 expression.

To the best of our knowledge, the present study demonstrated for the first time that USF1 expression was enhanced in cervical cancer tissues. As a ubiquitously expressed transcription factor, USF1 was able to bind the promoter region of p65 and transcriptionally activate the expression of p65, thereby enhancing the migration and invasion of cervical cancer cells.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

WW performed the experiments and analyzed the data. SY, HJ, JD, XC, XT and YG performed part of RT-qPCR. SZ designed the experiments, analyzed the data and gave final approval of the version to be published. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Review Board of Shandong University and written informed consent was obtained from all patients prior to their inclusion.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


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