Long non-coding RNA TP73 antisense RNA 1 facilitates the proliferation and migration of cervical cancer cells via regulating microRNA-607/cyclin D2

HONGMEI ZHANG1*, BING XUE2*, SHUYUAN WANG3, XIAOXIA LI2 and TINGTING FAN4

1Department of Pathology, Jining No. 1 People's Hospital, Jining, Shandong 272011; 2Department of Pathology, Affiliated Hospital of Jining Medical University, Jining, Shandong 272000; 3Department of Gynecology, Tai'an Tumour Prevention and Treatment Hospital, Tai'an, Shandong 271000; 4Department of Gynecology, People's Hospital of Chongqing Hechuan, Chongqing 401519, P.R. China

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Abstract. The present study aimed to explore the effect of the long non-coding RNA TP73 antisense RNA 1 (TP73-AS1) on cervical cancer progression. Cervical cancer and adjacent tissues were collected from 56 patients and assessed. In addition, HeLa and CaSki cells were transfected with various plasmids, inhibitors and corresponding controls, and then Cell Counting Kit-8 and Transwell assays were used to detect the cell proliferation, migration and invasion abilities. Luciferase reporter gene assay was also performed in HeLa cells. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to investigate TP73-AS1, microRNA-607 (miR-607) and cyclin D2 (CCND2) gene expression, while CCND2 protein expression was determined by western blot analysis. The results revealed that the TP73-AS1 level was upregulated in cervical cancer tissues (P<0.05) and predicted a poor 5-year overall survival (P<0.05). Hela and CaSki cells transfected with siTP73-AS1 exhibited reduced proliferation, migration and invasion abilities when compared with those in the siNC group (P<0.05). Furthermore, miR-607 was found to be negatively regulated by TP73-AS1, while CCND2 was negatively regulated by miR-607. HeLa and CaSki cells transfected with siTP73-AS1 exhibited lower CCND2 mRNA and protein expression levels compared with the siNC and siTP73-AS1 + miR-inhibitor groups (P<0.05). Compared with the siNC and siTP73-AS1 + CCND2 overexpression groups, siTP73-AS1-transfected HeLa and CaSki cells had decreased proliferation, migration and invasion abilities (P<0.05). In conclusion, the findings suggested that upregulation of TP73-AS1 promoted cervical cancer progression by promoting CCND2 via the suppression of miR-607 expression.

Introduction

According to the latest statistical data from the International Agency for Research on Cancer, there were 528,000 new cases of cervical cancer and 266,000 associated mortalities worldwide in 2012 (1). Cervical cancer is the most common gynecological malignant tumor that seriously threatens the health and life of women. The majority of cervical cancer cases are reported in developing countries, accounting for approximately 85% of the total number of patients worldwide (2). Despite advances in screening for cervical cancer, surgical techniques, radiotherapy and chemotherapy, the efficacy of clinical treatment of advanced and recurrent cervical cancer remains unsatisfactory.

With the development of modern molecular biology and genomics, targeted therapy has become one of the research hotspots for the treatment of advanced or recurrent cervical cancer (3). During the targeted therapy process, an effective therapeutic target enables the drug to specifically bind to the oncogenic site, thereby promoting the specific death of tumor cells. Thus, the discovery of therapeutic targets has become a research focus. Currently, in addition to coding genes, certain non-coding genes have also been identified as effective targets for the treatment of tumors, including cervical cancer. Accumulated studies have suggested that long non-coding RNAs (IncRNAs) exhibit an important role in the occurrence and progression of tumors (4,5). It has been reported that IncRNAs regulate gene expression by affecting epigenetic, transcriptional and post-transcriptional levels (4,5). Several IncRNAs have also been demonstrated to be effective therapeutic targets for cervical cancer. For instance, abnormal upregulation of the IncRNA RP11-396F22.1 was closely associated with poor prognosis of cervical cancer patients (6). In addition, Liu et al (7) revealed that the IncRNA SNHG1 was upregulated in cervical cancer, resulting in enhanced tumor cell
proliferation, migration and invasion. The lncRNA HOTAIR was also found to enhance cervical cancer cell proliferation and migration via inhibition of microRNA (miR)-326 (8). Furthermore, reduced lncRNA GAS5 level may be associated with poor prognosis of patients with cervical cancer (9,10). TP73 antisense RNA 1 (TP73-AS1) is a member of the lncRNA family, and its expression and effects in several tumors have been studied (11,12). However, whether TP73-AS1 regulates cervical cancer has not been reported to date. The present study aimed to explore the expression and influence of TP73-AS1 in cervical cancer, and further investigated the underlying molecular mechanisms to determine whether this lncRNA is an effective therapeutic target and provide a theoretical basis for the treatment of cervical cancer.

Materials and methods

Ethics statement. All patients voluntarily participated in the present study and signed informed consent. The study was performed with the approval of the Ethics Committee of People's Hospital of Chongqing Hechuan.

Patients and cervical cancer tissues. A total of 56 patients were involved in the present study, who were first diagnosed with cervical cancer between April 2011 and August 2015, and underwent surgery at People's Hospital of Chongqing Hechuan. During surgery, 56 tumor tissues and 56 corresponding adjacent normal tissues were obtained and stored in liquid nitrogen. Following surgery, all patients were followed up for 5 years, and the follow-up was completed in the case of mortality during this 5-year period. Based on the follow-up records, the 5-year overall survival rate of patients was analyzed by Kaplan-Meier survival analysis.

Cell culture. A human cervical epithelial cell line (HCVePC) and two cervical cancer cell lines (HeLa and CaSkii cells) were all provided by the Type Culture Collection of the Chinese Academy of Sciences. All cells were maintained in Dulbecco's Modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) in an incubator with 5% CO2 at 37°C. The medium was supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), as well as penicillin (100 U/ml) and streptomycin (100 U/ml).

Cell transfection. Small interfering RNA (siRNA) targeting TP73-AS1, miR-607 mimics, miR-607 inhibitors and the corresponding negative controls were synthetized by Thermo Fisher Scientific, Inc. (Invitrogen; Thermo Fisher Scientific, Inc.). For CCND2 overexpression, the coding sequence of the cyclin D2 (CCND2) gene was constructed into a pcDNA3 vector (Invitrogen; Thermo Fisher Scientific, Inc.). All transfections were performed using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol, at a concentration of 50 nM for siRNAs or miRNAs, and 1 µg/well for the pcDNA3 vector. After 48 h at 37°C, the transfection efficiency was validated using reverse transcription-quantitative polymerase chain reaction (RT-qPCR), as described later in the methods.

Cell proliferation detection by Cell Counting Kit-8 (CCK-8) assay. The transfected cells of each group were seeded into 96-well plates for routine culture at 37°C and 5% CO2, with each well containing 1x104 cells. After 24, 48 and 72 h of culture, 10 µl CCK-8 solution (Dojindo Molecular Technologies, Inc.) was added to each well. After 4 h of incubation, a microplate reader was used to detect the optical density (OD) value of each well at a wavelength of 450 nm.

Cell migration and invasion Transwell assays. A Transwell chamber was inserted into a 24-well plate containing 600 µl DMEM (10% FBS) in each well. Serum-free cell suspensions with a volume of 150 µl were added to the upper chamber of the Transwell chamber. For the cell invasion assay, the upper layer of the chamber was pre-coated with a thin layer of Matrigel. For the cell migration assay, a similar experiment was conducted, with the exception that Matrigel was not used. After 2 days, all chambers were removed and the residual liquid was discarded. Matrigel and any remaining cells in the upper layer of the chamber were gently wiped off with a wet cotton swab. Cells in the lower layer of the chamber were washed twice with PBS and fixed with 90% ethanol for 30 min. Crystal violet (0.1%) was subsequently used to stain cells for 30 min, and the invading or migrating cells were counted under a microscope in five random, nonoverlapping fields of view.

Luciferase reporter gene assay. The interaction between TP71-aS1 and miR-607 was predicted using the miRDB online database (http://www.mirdb.org/miRDB/index.html). The interaction between miR-607 and CCND2 was predicted using the TargetScan version 7.1 tool (http://www.targetscan.org/vert_71/). For the luciferase reporter assay, mutant (Mut) and wild-type (WT) sequences of TP73-AS1 or CCND2 were constructed into the pGL3-basic luciferase reporter vector (Promega Corporation). Subsequently, miR-607 mimics and reporters were transfected into HeLa cells. After 48 h, the relative luciferase activity was measured using a dual-luciferase reporter assay system (Promega Corporation) and normalized to the Renilla luciferase activity.

RT-qPCR assay. TRIzol reagent (Thermo Fisher Scientific, Inc.) was used to extract total RNA from the tissues and cells, strictly following the manufacturer's protocol. The RNA concentration was determined using a Nanodrop® 2000 spectrophotometer (Nanodrop Technologies; Thermo Fisher Scientific, Inc.) A total of 1 µg RNA samples were collected and used to perform an RT reaction with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). qPCR was then conducted with Fast Start Universal SYBR Green Master (Roche Diagnostics). The qPCR program was set as follows: 50°C for 2 min initially, followed by 95°C for 10 min, and 38 cycles of 95°C for 15 sec and 60°C for 1 min. The 2ΔΔCt method (13) was subsequently used as the common method for analyzing the relative gene expression. U6 and GAPDH served as the internal reference for miRNA and mRNA detection, respectively. Primer sequences were as follows: TP73-AS1, forward 5'-AGAGGTGAGTGAAATGGCTACC-3', reverse 5'-TGGACCCAGGAGAGGAT-3'; U6, forward 5'-AAGGAGACCCAGACAGAC-3', reverse 5'-GGAAATCTGGTAAAGCTTTCCA-3'; miR-607, forward 5'-AACGAGACACCAGACAGAC-3', reverse 5'-TGGGAATCCAGATCTATAAC-3'; CCND2, forward 5'-ACCT
Western blot analysis. Using RIPA buffer (Thermo Fisher Scientific, Inc.), total proteins were extracted from HeLa and Ca Ski cells, followed by determination of the protein concentrations using a BCA Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.). Next, 30 µg of each protein sample was used to conduct sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then the separated proteins were transferred to a PVDF membrane. Subsequent to blocking with 5% skimmed milk for 1 h at room temperature, the PVDF membrane was probed with anti-CCND2 primary antibody (1:1,000; cat. no. ab207604; Abcam) overnight at 4˚C. TBS-Tween-20 was used to wash the membrane prior to incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:5,000; cat. no. ab7090; Abcam). An enhanced chemiluminescence reagent (Thermo Fisher Scientific, Inc.) was used to detect the signals, followed by analysis using Image Lab 3.0 software (Bio-Rad Laboratories, Inc.). With GAPDH (1:1,000; cat. no. ab9485; Abcam) serving as the internal reference, the relative CCND2 protein expression was determined.

Statistical analysis. All data in this study were processed using SPSS software (version 19.0; IBM Corp.), and are expressed in the form of the mean ± standard deviation. Student's t-test was used to analyze differences between two groups, while one-way analysis of variance followed by Tukey's post hoc test was used to analyze differences among multiple groups for statistical significance. Pearson's correlation analysis was used to analyze the expression correlation between two genes. For survival rate analysis, the samples were divided into the low or high expression groups for TP73-aS1, mir-607 or CCND2 based on their median expression value. The Kaplan-Meier method was used to draw survival curves, and the log-rank test was used to determine statistical significance. All experiments were repeated three times independently. P<0.05 was set as the threshold for statistically significant differences.

Results

High expression of TP73-AS1 in cervical cancer tissues predicts poor 5-year overall survival. According to the results of RT-qPCR, TP73-AS1 expression was significantly increased in cervical cancer tissues as compared with the adjacent normal tissues (P<0.05; Fig. 1A). Subsequently, the current study further researched TP73-AS1 expression in cervical cancer cells through in vitro experiments. Compared with the normal HcvepC cells, HeLa and CaSki cervical cancer cells exhibited a significantly higher relative TP73-AS1 expression (P<0.05; Fig. 1B). Thus, these findings indicated that TP73-AS1 was upregulated in cervical cancer tissues and cells. The 5-year overall survival of patients was then analyzed by Kaplan-Meier survival analysis. As shown in Fig. 1C, patients with high TP73-AS1 expression exhibited a markedly reduced 5-year overall survival rate in comparison with those exhibiting low TP73-AS1 expression (P<0.05).

TP73-AS1 knockout hinders the proliferation, migration and invasion of cervical cancer cells. In subsequent experiments, HeLa and CaSki cells were transfected with TP73-AS1 siRNA (siTP73-AS1 group) or TP73-AS1 siRNA negative control (siNC group). As displayed in Fig. 2A, TP73-AS1 relative expression in cells of the siTP73-AS1 group was significantly lower compared with that in untreated or siNC-treated cells (P<0.05), revealing that TP73-AS1 expression in HeLa and CaSki cells was successfully downregulated by transfection with TP73-AS1 siRNA. Following the transfection, the proliferation ability of the two cell lines was measured by a CCK-8 assay. Compared with the siNC group, cells in the siTP73-AS1 group exhibited a markedly lower OD<sub>450</sub> value at 72 h, indicating significant reduction in proliferation (P<0.05; Fig. 2B and C). Furthermore, according to Transwell experiments, the relative number of migrating and invading cells in the siTP73-AS1 group also significantly declined when compared with that in the untreated and siNC groups (P<0.05; Fig. 2D and E). Taken together, TP73-AS1 knockout hindered the proliferation, migration and invasion of cervical cancer cells.
TP73-AS1 functions as a sponge for miR-607. Through bioinformatics analysis, it was observed that TP73-AS1 may function as a sponge for miR-607, since miR-607 achieved the highest score among all candidates. In addition, among all potential candidates, only miR-607 has previously been reported to inhibit tumor progression (14). Thus, only miR-607 was selected in the present study for further investigation.

Figure 2. TP73-AS1 knockout hindered the proliferation, migration and invasion of cervical cancer cells. (A) TP73-AS1 expression in HeLa and CaSki cells were successfully downregulated by transfection with TP73-AS1 siRNA. (B) HeLa and (C) CaSki cell proliferation, examined by CCK-8 assay. Compared with the siNC group, HeLa and CaSki cells in the siTP73-AS1 group exhibited significantly lower OD450 values at 72 h. (D) The relative number of migrating cells in the siTP73-AS1 group significantly declined when compared with that of the siNC group. (E) Significantly reduced relative number of invading cells was identified in the siTP73-AS1 group when compared with that of the siNC group. *P<0.05 vs. siNC group. TP73-AS1, TP73 antisense RNA 1; siRNA, small interfering RNA; NC, negative control; CCK-8, Cell Counting Kit-8.

Figure 3. TP73-AS1 is a sponge for miR-607. (A) Binding site of TP73-AS1 and miR-607 was analyzed. (B) Reverse transcription-quantitative polymerase chain reaction detection of miR-607 expression (left panel) and luciferase reporter gene assay (right panel), indicating that miR-607 suppressed the activity of WT TP73-AS1. (C) miR-607 relative expression in HeLa and CaSki cells of the siTP73-AS1 group was significantly higher than that of the siNC group. (D) TP73-AS1 and miR-607 relative expression levels were negatively correlated. (E) Relative expression of miR-607 in tumor and normal tissues. (F) Overall survival rate was analyzed based on miR-607 expression. *P<0.05 vs. miR-NC or siNC groups. TP73-AS1, TP73 antisense RNA 1; miR, microRNA; si-, small interfering RNA; NC, negative control; WT, wild-type; Mut, mutant.
of HeLa cells in the miR-nc and miR-607 groups. However, the insertion of the wild-type TP73-AS1 sequence significantly decreased the relative luciferase activity of HeLa cells in the miR-607-transfected group when compared with that in the miR-nc group (P<0.05; Fig. 3B). At the same time, miR-607 relative expression in HeLa and CaSki cells of the siTP73-AS1 group was significantly higher in comparison with that of the siNC group (P<0.05; Fig. 3C). Furthermore, there was a negative correlation between TP73-AS1 and miR-607 levels (r=-0.777; Fig. 3D). Besides, it was observed that miR-607 expression was significantly lower in normal tissues (Fig. 3E), while high expression of miR-607 predicted a high survival rate for cervical cancer patients (Fig. 3F). These data indicated that TP73-AS1 functions as a sponge for miR-607.

miR-607 directly inhibits CCND2 expression. The binding sites of CCND2 and miR-607 are listed in Fig. 4A. For HeLa cells in the miR-nc and miR-607 groups, there was no significant difference in relative luciferase activity following insertion of a CCND2 mutant sequence into the cells. However, CCND2 wild-type sequence insertion markedly reduced the relative luciferase activity of HeLa cells in the miR-607 group as compared with that in the miR-nc group (P<0.05; Fig. 4B), suggesting that CCND2 expression was directly inhibited by miR-607. Evidently lower CCND2 mRNA and protein expression levels were also observed in HeLa and CaSki cells of the miR-607 group when compared with the miR-nc group (P<0.05; Fig. 4C and D). Pearson's correlation analysis revealed a negative correlation between miR-607 and CCND2 mRNA expression (r=-0.802; Fig. 4E). Furthermore, CCND2 expression was found to be significantly higher in tumor tissues compared with the adjacent normal tissues (Fig. 4F), while high expression of CCND2 was associated with a lower survival rate (Fig. 4G).

Downregulation of TP73-AS1 suppresses CCND2 expression via promoting miR-607. Bioinformatics analysis was further used to search for the potential targets of miR-607, and the data identified that CCND2 achieved a very high score and was a classical oncogene (15). Thus, this gene was selected for subsequent investigation. As displayed in Fig. 5A, the insertion of the CCND2 mutant sequence did not affect the relative luciferase activity of HeLa cells in the siNC group and siTP73-AS1 groups. However, it was noted that CCND2 wild-type insertion significantly reduced the relative luciferase activity of HeLa cells in the siTP73-AS1 group when compared with that in the siNC group (P<0.05), indicating that TP73-AS1 knockout inhibited CCND2 expression. Subsequently, miR-607 inhibitor was used, and its transfection efficiency was confirmed, as shown in Fig. 5B. HeLa and CaSki cells were subjected to co-transfection with TP73-AS1 siRNA and miR-607 inhibitor (siTP73-AS1 + miR-inhibitor group). According to Fig. 5C and D, CCND2 mRNA and protein expression in HeLa and CaSki cells of the siTP73-AS1 group was markedly lower compared with that in the siNC and siTP73-AS1 + miR-inhibitor groups (P<0.05). However, no significant changes were observed in CCND2 mRNA and protein expression levels between the siNC and siTP73-AS1 + miR-inhibitor groups (Fig. 5C and D). These results illustrated that downregulation of TP73-AS1 suppressed CCND2 expression via promoting miR-607.

TP73-AS1 knockout hinders the proliferation, migration and invasion of cervical cancer cells via suppressing CCND2. Next, CCND2 control and overexpression vectors

![Figure 4](image-url)

**Figure 4.** CCND2 was directly suppressed by miR-607. (A) Binding site of CCND2 and miR-607 was predicted. (B) Luciferase reporter gene assay indicated that miR-607 suppressed the activity of WT CCND2. (C) mRNA and (D) protein CCND2 expression levels were decreased by miR-607 transfection. (E) Pearson's correlation analysis revealed a negative correlation between miR-607 and CCND2 mRNA expression. (F) Relative expression of CCND2 in tumor and normal tissues. (G) Overall survival rate was analyzed based on CCND2 expression. *P<0.05 vs. miR-nc group. CCND2, cyclin D2; miR, microRNA; NC, negative control; WT, wild-type; Mut, mutant.
were used to transfect HeLa and CaSki cells, referred to as the oeCtrl and oeCCND2 groups, respectively. Compared with the oeCtrl group, significantly elevated CCND2 mRNA expression was observed in HeLa and CaSki cells of the oeCCND2 group (P<0.05), revealing that HeLa and CaSki cells were successfully transfected (Fig. 6A).
Co-transfection with TP73-AS1 siRNA and CCND2 over-expression vectors was also performed in HeLa and CaSki cells (siTP73-AS1 + oeCCND2 group). HeLa and CaSki cells in the siTP73-AS1 group exhibited significantly lower relative proliferation, migration and invasion in comparison with those in the siNC and siTP73-AS1 + oeCCND2 groups (P<0.05; Fig. 6B-D). However, no evident difference was identified in the relative proliferation, migration and invasion of cells in the siTP73-AS1 + oeCCND2 group as compared with the siNC group (Fig. 6B-D).

Discussion

In the present study, it was observed that TP73-AS1 was aberrantly overexpressed in cervical cancer tissues and cells, which promoted cervical cancer progression by promoting CCND2 through the suppression of miR-607 expression. Researchers have recently reported that IncRNAs are closely associated with tumorigenesis (16). In patients with brain glioma, high TP73-AS1 expression was correlated with poor prognosis, and TP73-AS1 functioned as a carcinogenic IncRNA in this tumor, contributing to brain glioma cell invasion and proliferation (11). It was also demonstrated that downregulated TP73-AS1 inhibited breast cancer cell proliferation (12). A study by Li et al (17) revealed that ovarian cancer patients with higher TP73-AS1 expression exhibited lower survival when compared with those with lower TP73-AS1 expression. To the best of our knowledge, no study in the literature has thus far defined the role of TP73-AS1 in cervical cancer. Therefore, the present study is the first to discover that TP73-AS1 was upregulated in cervical cancer and promoted tumor progression by upregulating CCND2 through the inhibition of miR-607.

miRNAs are a class of non-coding small RNAs with a length of approximately 19-23 nucleotides, which are important post-transcriptional regulators in vivo and widely involved in various biological behaviors of cells (18). Studies have identified that miRNAs are closely associated with the biological processes of cervical cancer cells, such as proliferation, apoptosis, invasion and angiogenesis, which are considered to be new targets for the diagnosis, treatment and prognosis of this tumor (19). Several miRNAs exert a carcinogenic role in cervical cancer, such as miR-92, miR-150, miR-21, miR-494 and miR-155, among others (20-24). In addition, certain miRNAs serve as tumor suppressor in cervical cancer, including miR-138, miR-195, miR-362, miR-218, miR-744 and so on (25-29). The main factors associated with cervical cancer prognosis include age, pathological type, FIGO stage and lymph node metastasis (24,30,31). Recent studies have identified that miRNA expression levels are closely correlated with the FIGO stage, tumor differentiation, human papillomavirus (HPV) infection and lymph node metastasis, and may thus be used as prognostic indicators and independent prognostic factors for cervical cancer (24,30,31).

CCND2 is a cell cycle-associated gene that encodes the CCND2 protein. Abnormal cell cycle is an important early event leading to cervical cancer. In a previous study, researchers reported that CCND2, an important factor in regulating the transition from G1 to S phase in the cell cycle, was abnormally overexpressed in several tumors, including in cervical cancer (15). CCND2 protein can bind to cyclin-dependent kinase 4 (CDK4) and CDK6 to form a Cyclin/CDK complex. This complex then facilitates a series of processes that ultimately lead to tumor cells entering the DNA synthesis phase (32). Carcinogenic factors may cause abnormal expression of CCND2 protein, which leads to uncontrolled cell cycle, infinite proliferation of cells and loss of apoptotic ability, and ultimately promotes cell malignant transformation and tumor formation (33). The current study results indicated that CCND2 transcription and translation in cervical cancer was aberrantly activated by upregulated TP73-AS1, which enhanced the progression of this tumor.

However, the current study also has several limitations. For example, the correlation of TP73-AS1 expression with the clinical stage or HPV status of patients could not be examined. Furthermore, the effect of TP73-AS1 on cell cycle progression was not analyzed. Additionally, whether overexpression of TP73-AS1 in the normal cell line HCvePC was able to induce a cancer-like phenotype was not investigated in the present manuscript.

In conclusion, the present article first demonstrated that TP73-AS1 was abnormally overexpressed in cervical cancer, which promoted cervical cancer progression by promoting CCND2 via the suppression of miR-607 expression. Thus, TP73-AS1 may be considered as a novel target for cervical cancer treatment.

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Availability of data and materials

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

Authors' contributions

HZ and TF initiated and designed the study, analyzed and interpreted the results, and wrote the manuscript. HZ and BX performed the experiments. SW and XL interpreted the results, and wrote the manuscript. HZ and TF initiated and designed the study, analyzed and interpreted the results, and wrote the manuscript. HZ and BX participated in all the experiments. SW and XL performed western blotting. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Research Ethics Committee of Jining No. 1 People's Hospital, and all enrolled patients signed a written informed consent document.

Patient consent for publication

All patients included in this study provided consent for the publication of their data.
Competing interests

The authors declare that they have no competing interests.

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