The mTOR Pathway Regulates PKM2 to Affect Glycolysis in Esophageal Squamous Cell Carcinoma

He Xiaoyu, PhD¹, Yin Yiru, MD², Shi Shuisheng³, Cheng Keyan, PhD⁴, Yan Zixing, MD⁵, Cheng Shanglin, MD⁴, Wang Yuan, MD³, Cheng Dongming, MD¹, Zhang Wangliang, MD⁵, Bai Xudong, MD⁶, and Ma Jie, PhD¹

Abstract

Objectives: Esophageal squamous cell carcinoma is a highly prevalent cancer with poor survival rate and prognosis. Increasing evidence suggests an important role for metabolic regulation in treating esophageal squamous cell carcinoma, but the underlying mechanism remains unclear. The pyruvate kinase M2 isoform is a key enzyme in the energy production process, and the upregulation of pyruvate kinase M2 isoform also plays a crucial role in gene transcription and tumorigenesis. The mammalian target of rapamycin pathway regulates an array of cellular functions, including protein synthesis, metabolism, and cell proliferation. The pyruvate kinase M2 isoform and mammalian target of rapamycin pathways both affect metabolism in cancers, and evidence also suggests that the mammalian target of rapamycin downstream transcription factor hypoxia-inducible factor-1α regulates pyruvate kinase M2 isoform. We therefore investigated the regulatory mechanism among pyruvate kinase M2 isoform, mammalian target of rapamycin, and aerobic glycolysis in esophageal squamous cell carcinoma, hoping to prove that mammalian target of rapamycin pathway regulates pyruvate kinase M2 isoform to affect glycolysis in esophageal squamous cell carcinoma.

Methods: Immunohistochemical staining was used to compare pyruvate kinase M2 isoform and phospho-mammalian target of rapamycin expression in 30 human pathological esophageal squamous cell carcinoma sections and 30 nontumoral esophageal tissues. Short hairpin RNA was used to inhibit pyruvate kinase M2 isoform and activate mammalian target of rapamycin, after which we monitored changes in glucose consumption and lactate production. Finally, we determined the expression of pyruvate kinase M2 isoform and the mammalian target of rapamycin downstream transcription factor hypoxia-inducible factor-1α, as well as glucose consumption and lactate production, following the modification of mammalian target of rapamycin expression.

Results: Immunohistochemical staining showed that both phospho-mammalian target of rapamycin and pyruvate kinase M2 isoform expression were higher in esophageal squamous cell carcinoma than in nontumor tissues. Glucose consumption and lactate production measurements demonstrated that altering mammalian target of rapamycin and pyruvate kinase M2 isoform levels caused corresponding changes in glycolysis in esophageal squamous cell carcinoma cells. When mammalian target of rapamycin was activated or inhibited, expression of pyruvate kinase M2 isoform and hypoxia-inducible factor-1α as well as glycolysis were altered, indicating that mammalian target of rapamycin regulates pyruvate kinase M2 isoform via the downstream transcription factor hypoxia-inducible factor-1α, thereby affecting glycolysis in esophageal squamous cell carcinoma. Conclusion: Mammalian target of rapamycin pathway promotes aerobic glycolysis in esophageal squamous cell carcinoma by upregulating pyruvate kinase M2 isoform. Both proteins can serve as molecular targets for novel therapeutic strategies.

1 Cardiothoracic Surgery, The Second Hospital of Shanxi Medical University, Taiyuan, China
2 Translational Medicine Research Center, Key Laboratory of Cellular Physiology, Ministry of Education, Shanxi Medical University, Taiyuan, China
3 Endoscopy Center, The Second Hospital of Shanxi Medical University, Taiyuan, China
4 Gynecology and Obstetrics, The Second Hospital of Shanxi Medical University, Taiyuan, China
5 Department of Pathology, The Second Hospital of Shanxi Medical University, Taiyuan, China
6 Cardiothoracic Surgery, Shanxi Academy of Medical Sciences, Shanxi Dayi Hospital, China

Corresponding Author:
Ma Jie, Cardiothoracic Surgery, The Second Hospital of Shanxi Medical University, No 382, Wuyi Road, Taiyuan, Shanxi, China.
Email: majie2012@163.com
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Abbreviations
EC, esophageal carcinoma; ESCC, esophageal squamous cell carcinoma; HC, hepatocellular carcinoma; HIF-1α, hypoxia-inducible factor-1α; mTOR, mammalian target of rapamycin; PKM2, pyruvate kinase M2 isoform; PI3K, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homolog deleted on chromosome ten; shRNA, short hairpin RNA.

Introduction
Esophageal cancer (EC) is ranked as the eighth most prevalent cancer and is the sixth leading cause of cancer-related deaths worldwide.1 The EC is among the leading causes of cancer death in China, with an estimated 4 292 000 new cancer cases and 2 814 000 cancer deaths occurred in China in 2015.2 Esophageal squamous cell carcinoma (ESCC) is one of the primary pathological subtypes of EC. The ESCC is predominant in East Asia and accounts for 95% of all Chinese patients with EC,3,4 showing poor survival,5 and considerable efforts have been made to detect the mechanisms that underlie the genesis, development, invasion, and metastasis of ESCC.

Mammalian target of rapamycin (mTOR) is a serine/threonine kinase that is activated by several oncogenic signaling pathways and is accordingly hyperactive in a majority of cancers,6 serving as a core kinase.7 The mTOR belongs to the PI3K family and regulates an array of cellular functions, including protein synthesis, metabolism, and cell proliferation. It is found in 2 discrete complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), which each contain distinct protein components and phosphorylate different substrates. The mTORC1 is acutely inhibited by rapamycin, which has been used therapeutically and experimentally as a probe to gain insight into mTORC1 regulation and function.8 The mTORC2 is significantly less sensitive to rapamycin than mTORC1.9 mTORC1 is known to suppress autophagy, which is a key catabolic process.10 The mTOR is phosphorylated by Akt1 at Ser2448 to yield phospho-mTOR (p-mTOR), which regulates ribosome biogenesis and protein synthesis,11 and is also present in the cytoplasm and nucleus, with high p-mTOR expression indicating reduced response to tumor treatment.12,13

Rapamycin is a potent and specific mTOR inhibitor.14,15 The mTOR signaling pathway can also be suppressed by genes, such as tuberous sclerosis complex 2 (TSC2)16,17 and phosphatase and tensin homolog deleted on chromosome ten (PTEN).16 The loss or mutation of these genes leads to a wide variety of human sporadic and inherited cancer. We have observed increased activity of the mTOR pathway after silencing PTEN.18 In the present study, we knocked down PTEN to activate the mTOR pathway.

Hypoxia-inducible factor-1α (HIF-1α), a downstream target of mTOR, is a major transcription factor that controls cellular adaptation to hypoxia and can influence glycolytic metabolism under not only hypoxic but also normoxic conditions.19,20 The HIF-1α triggers the expression of glycolytic markers that influence the glycolytic rate in tumor cells. These alterations increase tumor cell proliferation (Warburg effect).21 Moreover, knocking down HIF-1α inhibits lactate production and pyruvate kinase M2 isoform (PKM2) expression in pancreatic cancer.22

In 1956, Otto Warburg discovered that cancer cells tend to convert glucose into lactate to produce energy under oxygenated conditions, which is termed aerobic glycolysis or the Warburg effect.23 The PKM2 is an important cancer metabolism enzyme that is responsible for the Warburg effect. In highly glycolytic cancers, pyruvate is converted to lactic acid and adenosine triphosphate to fuel rapid cellular proliferation.24 Enhanced PKM2 expression is frequently observed in both the cytoplasm and the nucleus in various human cancers,25 such as pancreatic cancer, hepatocellular carcinoma,26 colorectal cancer,25 and cervical cancer,27 and is also important for tumor growth.28 Sun et al28 reported that mTOR-induced PKM2 expression in mouse kidney tumors is mediated by HIF-1α and c-Myc,27 which was previously found to interact with an associated regulatory protein of mTOR.20,29

We hypothesized that knocking down PTEN will affect mTOR and thus regulate PKM2, inducing cancer cells to convert glucose into lactic acid to produce energy, which may be the major pathway for cellular energy metabolism in ESCC.

Materials and Methods
Study Population
The study was approved by the local Ethics Committee (2018 no 001), and informed consent was obtained from all patients. All procedures involving human specimens were performed with written informed consent according to the Declaration of Helsinki. The ESCC tissues from patients absent any radiation and chemotherapy who had pathologically confirmed disease between January 2004 and November 2016 at The Second Hospital of Shanxi Medical University were eligible for this study. The tumor group comprised 15 male and 15 female patients aged 28 to 75 years.
Concurrently, we selected 30 (15 males and 15 females) nontumoral tissue samples for the control group. The control and tumor group showed no significant differences in terms of age (control group: 60.5 [11.5]; tumor group: 59.2 [7.4], t = 0.548, P = .586). In the tumor group, tumor staging ranged as follows: I, 8/30 (26.7%); II, 13/30 (43.3%); III, 9/30 (30%); and IV, 0/30 (0%).

**Immunohistochemistry Analysis**

Tissue blocks were subjected to routine hematoxylin and eosin staining to confirm the diagnosis. Sections from the tissue blocks were sliced at 4-μm thickness, mounted on slides, subjected to antigen retrieval, coated with poly-L-lysine, and treated overnight with the following primary antibodies at 4°C: anti-PKM2 antibody (1:500, PKM2 [D78A4] XP Rabbit mAb #4053, Cell Signaling Technology, USA) and anti-p-mTOR antibody (1:200, Anti-mTOR [phospho-S2448], rabbit polyclonal, Abcam ab84400). The sections were then treated for 1 hour at room temperature with goat antirabbit immunoglobulin G (IgG; H+L) secondary antibody conjugated to horseradish peroxidase (1:1000, Novex A16104SAMPLE; Life Technologies, New Zealand), followed by incubation with ABC peroxidase for 1 hour, staining with diaminobenzidine for 5 minutes and counterstaining with hematoxylin (Gene Tech, China). As a negative control, the primary antibody was replaced with phosphate buffer saline (PBS). All sections were assessed at 200× magnification via light microscopy as follows. We quantitatively scored the tissue sections according to the percentage of positive cells and staining intensity, as described below.30 We rated the intensity of staining on a scale of 0 to 3: 0, negative; 1, weak; 2, moderate; and 3, strong. We assigned the following proportion scores: 0 if 0% of the tumor cells showed positive staining, 1 if 0% to 1% of the cells were stained, 2 if 2% to 10% were stained, 3 if 11% to 30% were stained, 4 if 31% to 70% were stained, and 5 if 71% to 100% were stained. We then combined the proportion and intensity scores to obtain a total score (range: 0-8) as described previously.30 The results were assessed by 2 experienced pathologists in a blinded manner.

**Cell Culture**

The human ESCL cell line KYSE150 was obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and maintained in modified RPMI medium (HyClone™, SH30809.01; Logon, Utah) supplemented with 10% fetal bovine serum (Gibco 1009141, Australia origin) and 1% penicillin-streptomycin (Solarbio P1400, China). The cells were used for experiments when the cells reached 60% confluency.

**Rapamycin Assay and Coimmunofluorescence**

KYSE150 cells were incubated with rapamycin (VETEC V900930, Sigma, USA) at 0, 10, 20, 50, or 100 nmol/L for 0, 2, 6, 12, or 24 hours to determine the inhibition of mTOR and p-mTOR by communofluorescence. After the cells were fixed with paraformaldehyde, they were incubated with primary antibodies against mTOR (anti-mTOR antibody [53E11], mouse monoclonal, Abcam ab87540, USA) and p-mTOR (anti-mTOR [phospho-S2448], rabbit polyclonal, Abcam ab84400, USA) followed by treatment with secondary antibodies (donkey anti-mouse IgG [H+L] highly cross-adsorbed secondary antibody, Alexa Fluor 488; donkey anti-rabbit secondary antibody, Alexa Fluor 594; Invitrogen, USA). Fluorescence was observed on an Olympus IX 71 (Olympus, Japan) microscope.

**Modification of PKM2 and mTOR Expression**

The PKM2 gene was knocked down based on the target sequence gcatctgtagtgaataaa using OmicsLink short hairpin RNA (shRNA) expression clone (FulenGen HSH013185-CH1, GeneCopeoeia®, Guangzhou, China). The PKM2 gene was overexpressed using OmicsLink expression clone (FulenGen EX-Z7438-M29, GeneCopeoeia, NM_002654). Both constructs were transfected into cells using the Lipofectamine 2000 transfection reagent (Invitrogen, 11668027). The PTEN gene was also knocked down using OmicsLink shRNA expression clone (FulenGen HSH015535-CH1, GeneCopeoeia) with Lipofecta
time 2000 transfection reagent. Transfection was conducted for 48 hours, after which successful transfection was defined as at least 70% of the cells exhibiting green fluorescence.

Quantitative polymerase chain reaction (qPCR) was used to confirm the transfection of the appropriate plasmids. Total RNA was extracted from KYSE150 cells using an RNA simple total RNA kit (TIANGEN, DP419, China) according to the manufacturer’s instructions and then reverse-transcribed and subjected to real-time (RT) PCR analysis to examine PKM2 and PTEN expression levels. The reverse transcription reactions were performed on a Takara RR047A, whereas the qPCR reactions were performed on a Takara RR820A (One-Step SYBR PrimeScript RT-PCR kit) using the StepOnePlus qPCR system (Thermo Fisher, USA) and the following primers:

- GAPDH: FP, 5'-ACAACTTTTGGATATCGTGGAAGG-3' and RP, 5'-GCAATCATCGCCACGAGTTTTC-3'; PKM2: FP, 5'-TCGGGGCTGAAGGCAGTGATGTG-3' and RP, 5'-AGGGCGGCGAGGTTTCTCAAATA-3'; PTEN: FP, 5'-GAGGCTGACGATGTTTCTCAAATA-3' and RP, 5'-GGATTTGACGGCTCCTCCTACGTC-3'.

Data showed the target sequence gcatctgtagtgaataaa for PKM2 shRNA and the target sequence ggcgttacagaaacattat for PTEN shRNA.

**Western Blotting**

The cells were washed with PBS, then lysed in RIPA buffer containing protease inhibitors 1% phenylmethylsulfonyl fluoride (PMFSF). Proteins were harvested by centrifugation and then 45 μg protein from different samples were separated by 8% sodium dodecyl sulfate-polyacrylamide gels (Solarbio P1200) electrophoresis and then transferred to polyvinylidene
fluoride membranes using a Bio-Rad wet-transfer apparatus. The membranes were blocked with 5% bovine serum albumin in Tris-buffered saline/0.1% Tween 20 for 1 hour at room temperature and then incubated overnight at 4°C with the primary antibodies. Immunoblot analysis was performed using secondary antibodies conjugated to horseradish peroxidase, and the primary antibodies included anti-mTOR, anti-p-mTOR, anti-PKM2, and anti-GAPDH (Sangon Biotech, D110016, China). The blots were visualized using a Bio-Rad Western blot detection system.

**Lactate Assay**

Lactic acid from the culture media of KYSE150 cells in (1) the PKM2-downregulation group, (2) the PKM2-overexpression group, (3) the PTEN-regulation group (as an mTOR activation group), and (4) the rapamycin group was measured using a BioVision lactate colorimetric assay kit II (#K627-100) and a Spectra-Max M2 MultiMode microplate reader (Molecular Devices, Sunnyvale, California) at 450 nm.

**Statistical Analysis**

Unless otherwise stated, the results presented as the mean (standard deviation) of at least 3 independent experiments. Statistical significance was measured using Student t test, where P < .01 was deemed significant. An appropriate posttest has been applied for internal comparisons. All data were analyzed using SPSS 22.0 (SPSS, Inc, Chicago).

**Results**

**Both PKM2 and mTOR Are More Highly Expressed in Samples From Patients With ESCC**

Immunohistochemical analyses (Figure 1) showed that the p-mTOR expression score in the tumor group was 7.00 (0.25) in the cytoplasm and nucleus, which was significantly higher than that in the control group (4.00 [1.00], μ = 6.73, P < .01). Similarly, PKM2 expression in the tumor group was 7.00 (1.00) in the cytoplasm and nucleus compared to 4.00 (2.00) in the control group (μ = 6.81, P < .01). Thus, both p-mTOR and PKM2 are overexpressed in tumor tissues, which have important implications in the regulation of glycolysis in ESCC.

The control and tumor groups did not show a normal distribution and were described as the median and interquartile range, and the Wilcoxon rank-sum test was used to determine statistical significance. The positive rates of Bartonella in different areas and genders were analyzed using the χ² test. P < .01 was considered statistically significant (Figure 1).

**Modification of PKM2 Regulates Metabolism in an ESCC Cell Line**

In the KYSE150 ESCC cell line, the PKM2 gene was overexpressed and knocked down in different groups using shRNA. Western blotting confirmed the modification of PKM2 expression (Figure 2A). The remaining glucose in the medium showed that the PKM2-up and PKM2-down groups consumed...
more and less glucose, respectively, than the control group (Figure 2B). Lactate assays showed that modifying PKM2 expression also affected lactate production. The PKM2-up group produced more lactate and the PKM2-down group produced less lactate than the control group (Figure 2C). These results indicated that a high level of PKM2 increased glycolysis, whereas a low level of PKM2 inhibited glycolysis.

**The mTOR Pathway Regulates Metabolism in an ESCC Cell Line**

As shown in Figure 3A, the rapamycin-treated group exhibited downregulation of mTOR. Immunofluorescence assays showed that in the cells treated with 20 or 50 nmol/L rapamycin for 12 hours, p-mTOR was inhibited. We selected the cells incubated with 50 nmol/L rapamycin as the rapamycin group, aiming to inhibit mTOR and p-mTOR expression. Western blotting revealed that rapamycin inhibited p-mTOR protein levels (Figure 3B).

Measurement of the remaining glucose in the medium showed that the rapamycin group consumed less glucose than did the control group (Figure 3C). Furthermore, in the lactate assay, the rapamycin group produced less lactate than did the control group (Figure 3D). These results indicate that inhibition of the mTOR pathway resulted in the generation of less lactate and that the mTOR pathway regulates glycolysis.

The amino acid sequence of mTOR is too long to be over-expressed in ESCC cells such as KYSE150. However, the *PTEN* gene is a key suppressor gene in the mTOR pathway, as mentioned earlier; therefore, we knocked down PTEN to activate mTOR. The *PTEN* gene was knocked down using shRNA to activate the mTOR pathway, and the modified PTEN expression was confirmed by qPCR. Western blotting showed that knocking down PTEN was associated with increased p-mTOR expression (Figure 3B).

The remaining glucose in the medium showed that the PTEN-down group consumed more glucose than did the control group (Figure 3C), and in the lactate assay, the PTEN-down group produced more lactate than did the control group.
Figure 3. mTOR regulates metabolism in an ESCC cell line. A, Fluorescence staining of KYSE150 cells treated with rapamycin. a1, a2, a3, a4: control group; b1, b2, b3, b4: rapamycin-treated cells (20 nM) after 12 hours; c1, c2, c3, c4: rapamycin-treated cells (50 nM) after 12 hours. Blue fluorescence shows the cell nucleus, green fluorescence indicates mTOR, and red fluorescence indicates p-mTOR. B, Western blot of p-mTOR.
The mTOR Pathway Regulates PKM2 to Affect Metabolism

Compared to the control group, PKM2 levels in the rapamycin group were downregulated (Figure 4A) and those in the PTEN-down group were upregulated (Figure 4A). These results showed that PKM2 was inhibited when the mTOR pathway was inhibited, whereas PKM2 was overexpressed when the mTOR pathway was activated. The glucose and lactate assay results shown in Figures 2 and 3 indicate that activation of the mTOR pathway had a similar effect on glycolysis as that of PKM2 overexpression.

We knocked down both PTEN and PKM2 in the same group (PTEN-down + PKM2-down group) to activate mTOR and inhibit PKM2. The glucose and lactate assays showed that glucose consumption (Figure 4B) and lactate production (Figure 4C) did not differ between this group and the PKM2-down group, indicating that activation of mTOR cannot increase aerobic glycolysis if PKM2 is knocked down in ESCC.

We found that activating mTOR regulates PKM2 to induce the Warburg effect in KYSE150 cells. Inhibitors of mTOR (including rapamycin derivatives) and PKM2 are thus potential drugs that could serve as new therapeutic options for cancer treatment (Figure 4B).

Discussion

PKM2 acts as a key enzyme for aerobic glycolysis. The mTOR pathway also plays a crucial role in tumors. In our study, we provided the first evidence that PKM2 is regulated by the mTOR pathway in ESCC. First, PKM2 and mTOR were both overexpressed in ESCC tissues, as determined by immunohistochemical analysis. Second, the results of glycolysis tests after alteration in PKM2 and mTOR expression confirm that the mTOR pathway regulated PKM2 to affect glycolysis in ESCC. These studies confirm the correlation between PKM2 and mTOR and indicate the possible mechanism underlying their relationship, which is crucial to affect glycolysis in ESCC.

PKM2 and mTOR are both important signaling molecules in cancer. Sun et al found that the mTOR pathway was able to affect PKM2 in human pancreatic (PANC-1), prostate (PC3), and liver (HepG2) cancer cell lines. Our immunohistochemical staining data suggest that PKM2 and p-mTOR are both overexpressed in ESCC tissues relative to their expression levels in nontumoral tissues. mTOR is phosphorylated by Akt1 at Ser2448, leading to downstream signaling by p-mTOR. The premise of our study was to examine the regulatory relationship between the mTOR pathway and PKM2.

PKM2 is considered an important enzyme that converts phosphoenolpyruvate to pyruvate in glycolysis. Lactate is one of the main products of cell aerobic glycolysis, especially in cancer, and glucose is metabolized to lactate through glycolysis. We suggested that the consumption of glucose and the production of lactate reflect the efficiency of cancer aerobic glycolysis. We knocked down or overexpressed the PKM2 gene using OmicsLink shRNA. The consumption of glucose and the production of lactic acid by ESCC cells changed following the modification of PKM2 expression. Gu et al and David et al found similar trends in other cancers. The results indicate that the regulation of PKM2 can induce changes in aerobic glycolysis.

The mTOR signaling pathway plays a central role in a wide spectrum of cellular activities, including cell proliferation, survival, and differentiation. This pathway also acts as a cellular rheostat that integrates the signals from a variety of cellular signaling pathways to sense growth factors, nutrient availability, and energy status. Studies by Sun et al showed that PKM2 is an important glycolytic enzyme in the oncogenic mTOR-induced Warburg effect. The HIF-1α and c-Myc-hnRNP cascades are the transducers of mTOR regulation of PKM2 in mouse kidney tumors. Zhou et al showed that pharmacological inhibition of mTOR signaling with rapamycin decreased the glycolytic capacity of 6 hepatocellular carcinoma cell lines. mTOR is now recognized as one of the major molecules in the pathway responsible for chemoresistance, which is activated in several oncogenic signaling pathways and can be activated by many molecules in cancer. We thus knocked down PTEN to activate mTOR and inhibited mTOR with rapamycin. Western blotting showed that HIF-1α changes according to mTOR expression. We performed tissue sectioning and Western blotting, which indicated that PKM2 also changes according to the expression of mTOR. This result suggests that the mTOR pathway regulates the expression of PKM2 in ESCC. The glucose assay and lactate assay showed that glycolysis changed with the level of mTOR and the regulation of PKM2. We conclude that the mTOR signaling pathway can regulate PKM2 in aerobic glycolysis to affect ESCC cells.

The PKM2 and mTOR pathways are known to be important signaling nodes, and drugs related to these pathways are undergoing laboratory tests and clinical trials for cancer treatment. Tang et al found that metformin was able to inhibit PKM2 through the mTOR pathway, indicating that PKM2 is an important signal molecule. As described above, PKM2 is not only
associated with disease progression and invasion but also involved in glycolysis.\textsuperscript{26} Drugs that inhibit enzyme sensitivity to substrates could also be considered for treatment. New therapies are needed to treat tumors, which are still a crucial health issue in the world. The mechanism of aerobic glycolysis draws our attention once again. Our experiments prove that mTOR can regulate PKM2 in ESCC, forming the basis for further exploring aerobic glycolysis, including through animal experiments \textit{in vivo}. In addition, survival time and individualized therapy plans should be taken into consideration. Our research may provide new insights into cancer treatment and suggests that a multitarget approach be applied to obtain multiple benefits.

**Authors’ Note**
He X, Ma J, Shi S, and Cheng K conceived and designed the experiments. He X and Yin performed the biochemical experiments. He X and Yin analyzed the data. Yan Z, Cheng S, Wang Y, Cheng D, and Bai X collected clinical information and tissue samples. Zhang W performed the immunohistochemical staining assay. He X wrote the article.

**Declaration of Conflicting Interests**
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**Ethical Approval**
The study was approved by The Shanxi Medical University Ethics Committee (2018 no 001), and informed consent was obtained from all the patients. All of the procedures involving human specimens were performed with written informed consent according to the Declaration of Helsinki.
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