AMP-activated protein kinase α1 serves a carcinogenic role via regulation of vascular endothelial growth factor expression in patients with non-small cell lung cancer

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Abstract. AMP-activated protein kinase α1 (AMPK α1) is involved in the tumorigenesis of various cancer types. However, the role of AMPK α1 in non-small cell lung cancer (NSCLC) remains unclear. In the present study, 99 NSCLC tumor tissues and paired normal tissues were obtained. The expression levels of AMPK α1 were significantly upregulated in NSCLC tumor tissues compared with those in adjacent non-tumor lung tissues. The patients were further divided into two groups according to their expression levels of AMPK α1 in tumor tissues. The results outlined that overexpression of AMPK α1 was associated with poor prognosis. In addition, vascular endothelial growth factor (VEGF) expression levels were associated with malignant progression in patients with NSCLC. Patients with NSCLC that overexpressed AMPK α1 and VEGF had the worst outcomes. Moreover, AMPK α1 may positively regulate VEGF expression. These results suggest that AMPK α1 serves a carcinogenic role at least in part through the regulation of VEGF expression, and thus represents a potential treatment target in patients with NSCLC.

Introduction

Malignant cells are characterized by uncontrolled growth and distant metastasis. An abundant energy supply is the foundation for cancer cell growth and metastasis, and as a result, metabolism is enhanced in cancer cells. As the primary regulator of cellular energy, AMP-activated protein kinase (AMPK) not only serves an important role in energy metabolism, but is also involved in cancer biology. AMPK is involved in tumorigenesis via the promotion of epithelial mesenchymal transition (1), regulation of cell growth and maintenance of tumor cell survival in numerous types of cancer. AMPK also has the potential to be a therapeutic target for cancer treatment (2,3). However, whether AMPK acts as an oncogene to promote cancer initiation and progression or a tumor suppressor is a matter of debate.

AMPK is a trimeric protein, and each subunit or isoform may have particular functions in different cancer types, which may account for the paradoxical effects of AMPK during tumorigenesis. Thus, it is necessary to demonstrate the role and mechanism of the AMPK isoforms separately. AMPK α1 (also known as protein kinase AMP-activated catalytic subunit α1 and encoded by the *PRKAA1* gene), is the most catalytic isoform of AMPK, and is predominantly expressed in the cytoplasm (4). Reportedly, the expression level of AMPK α1 was markedly increased in cancerous tissues compared with paired adjacent normal tissues, and the protein was preferentially expressed in the less differentiated areas of tumors (5). Furthermore, the proliferative ability of tumors was suppressed when AMPK α1 was inhibited (6). These findings indicated an oncogenic role for AMPK α1.

The present study was undertaken to investigate the role of AMPK α1 in non-small cell lung cancer (NSCLC). Tissue microarrays were used to determine the expression level of AMPK α1 in tumor tissues and adjacent normal tissues. Furthermore, lentiviruses were used to modulate the

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expression level of AMPK α1 *in vitro*. This aimed to determine whether AMPK α1 acts in a malignant manner in NSCLC, and ultimately the target of AMPK α1

Materials and methods

Patients and tissue samples. A total of 99 clinical NSCLC specimens were assessed. The tumor tissues and matched adjacent non-tumor tissues were collected from patients who had undergone surgical treatment at Subei People's Hospital (Yangzhou, China) between May 2004 and October 2012. The mean age of patients was 36-84 years (62.46±9.59) and patient information is presented in Table I. The present study was approved by the ethics board of Subei People's Hospital, and informed consent was obtained from all participants. Fresh samples were paraffin-embedded and verified by pathological examination following hematoxylin and eosin staining. Tissues were fixed with 10% formalin for 24 h at room temperature and paraffin embedded. Samples were sliced into 4-μm sections, which were stained with hematoxylin and eosin at room temperature for 10 and 2 min, respectively. Samples were examined by two pathologists who had no prior knowledge of the patients' clinicopathological information, using a light microscope in three fields and with a x400 magnification.

Cell culture. The human lung adenocarcinoma cell line A549 (Yingrun Biotechnologies Inc., Changsha, China) was used for experiments *in vitro*. Parental A549 cells and lentivirus-transfected cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel). Cells were maintained at 37°C in a humidified atmosphere (5% CO_2).

Tissue microarray (TMA) and immunohistochemistry (IHC). A total of 99 pairs of NSCLC tumor tissue samples and adjacent non-tumor tissue samples were fixed with 10% formalin for 24 h at room temperature and paraffin embedded to obtain the TMAs. Using biopsy needles, tissue cores (diameter, 3 mm) were obtained and placed on a recipient block. IHC was carried out using streptavidin-biotin-peroxidase complex method. TMA slides for IHC were deparaffinized by bathing in xylene solution for 10 min, and rehydrated through decreasing ethanol gradient (100, 90, 75 and 50%) for 5 min. Antigen retrieval was performed by incubating slides with citrate buffer (pH 6.0) at 95°C for 15 min. Endogenous peroxidase activity was blocked by incubating slides in 3% H_2O_2 at room temperature for 15 min. The slides were then incubated with primary antibodies against AMPK α1 (1:100; cat. no. D63G4; Cell Signaling Technology, Inc., Danvers, MA, USA) and VEGFA (1:100; cat. no. WL00009b; Wanleibio Co., Ltd., Shanghai, China) overnight at 4°C. The slides were incubated with biotin-labeled anti-rabbit secondary antibodies (1:100; cat. no. A0279; Beyotime Institute of Biotechnology, Haimen, China) at room temperature for 30 min, and detected with 3,3-diaminobenzidine (1:300; cat. no. P0203; Beyotime Institute of Biotechnology). Staining intensity and extent were examined using a light microscope at x400 magnification. Staining intensity was scored as 0, 1, 2 or 3 for negative, weak, moderate or strong staining, respectively. The extent of staining was scored based on the percentage of AMPK α1- or

Table I. Clinicopathological characteristics of patients with non-small cell lung cancer.

Variables	Cases, n	Cases, %
Age (years)		
<60	37	34.3
≥60	62	65.7
Sex		
Male	73	73.7
Female	26	26.3
Histotype		
Adenocarcinoma	51	51.5
Squamous cell	48	48.5
Primary tumor		
T1	21	21.2
T2	54	54.5
T3	17	17.2
T4	7	7.1
Regional lymph node		
N0	67	67.7
N1	19	19.2
N2	11	11.1
N3	2	2.0
Tumor-node-metastasis stage		
I-II	58	58.6
III-IV	41	41.4

VEGF-positive tumor cells. The final score was obtained by summing the scores for staining intensity and extent of staining.

Lentivirus and cell infection. To modulate AMPK α1 expression *in vitro*, lentiviruses, including LV-PRKAA1-RNAi (expressing a short interfering RNA targeting the *PRKAA1* gene; 5'-CTTTGCTTCTCTTATAAGTTATTGTGA-3'), LV-PRKAA1 (a lentivirus overexpressing the *PRKAA1* gene), and a negative LV-control were purchased from Shanghai GeneChem Co., Ltd. (Shanghai, China). A549 cells were infected with lentivirus according to the manufacturer's protocol. Briefly, $5x10^4$ cells supplemented with lentivirus (multiplicity of infection, 10) and 1 μl Polybrene (Shanghai GeneChem Co., Ltd.) were grown on 24-well plates. The supernatant was removed after 24 h and fresh culture medium was added to the cells. Fluorescence microscopy was used to observe the infection rate 72 h postinfection, and stable clones were selected after 2 weeks using puromycin (2 μg/ml) (GE Healthcare Life Sciences, Little Chalfont, UK). Western blotting was conducted to confirm the final infection efficiency.

Western blotting. Cells were harvested and lysed with radio-immunoprecipitation assay buffer (cat. no. P0013B; Beyotime Institute of Biotechnology) and the protein concentration was measured using a bicinchoninic acid assay. Sample concentrations were equalized, and proteins (29 μg, Fig. 4A;

15 μg, Fig. 4B) were separated by SDS-PAGE (10% gels). The proteins were subsequently transferred to polyvinylidene fluoride membranes and blocked with 5% skimmed milk in Tris-buffered saline for 2 h at room temperature. The membranes were incubated with primary antibodies against AMPK α1 (1:1,000; cat. no. D63G4; Cell Signaling Technology, Inc.) and vascular endothelial growth factor A (VEGFA; 1:500; cat. no. WL00009b; Wanleibio Co., Ltd) overnight at 4˚C, prior to incubation with a secondary horseradish peroxidase-labeled goat anti-rabbit antibody (1:1,000; cat. no. KGAA35; Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) for 1 h at room temperature. Bands were visualized using BeyoECL Plus (Beyotime Institute of Biotechnology). The primary antibody against GAPDH, (1:1,000, cat. no. 14C10; Cell Signaling Technology, Inc.) was used as an internal control. Densitometric analysis was performed using Image J v1.50i Software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Statistical analysis was performed using SPSS 22.0 (IBM Corp., Armonk, NY, USA). Data were expressed as the mean ± standard deviation, and analyzed using the Student's t-test. A Chi-square test was used to evaluate the association between AMPK α1 and VEGF. Kaplan-Meier curves were generated to assess overall survival (OS). Patients were classified based on the AMPK α1 or VEGF expression level in their tumor tissue into high expression (≥ median; n=49) or low expression (< median; n=50) groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Patients characteristics. The characteristics of the 99 surgically resected NSCLC tumors (51 adenocarcinoma and 48 squamous cell carcinoma samples) are displayed in Table I. Elderly patients (≥60 years old) accounted for 65.7%, of which 73 were male (73.7%) and 26 female (26.3%). The proportion of patients with early and late stage NSCLC was 58.6 and 41.4%, respectively.

High expression of AMPK α1 is associated with poor prognosis in NSCLC. The expression levels of AMPK α1 in NSCLC tumor tissues were compared with those in adjacent non-tumor lung tissues; AMPK α1 expression levels were significantly higher in tumor tissues compared with those in the adjacent tissues (Fig. 1A). The Kaplan-Meier method was used to determine the association between AMPK α1 expression level and prognosis. Patients with high AMPK α1 expression levels had a shorter OS time compared with patients with low AMPK α1 levels (Fig. 1B). According to these results, AMPK α1 may serve an aggressive role in NSCLC, and high levels of AMPK α1 may act as an indicator of poor prognosis in patients with NSCLC.

VEGF expression levels are associated with prognosis in patients with NSCLC. Angiogenesis has a prominent role during cancer occurrence, development, and metastasis. VEGF, an angiogenesis-driving factor, influences malignancy and is associated with poor prognosis in various types of cancer (7,8). Therefore, the association between VEGF and AMPK α1 levels in NSCLC were investigated. Consistent with previous reports (9,10), it was concluded that VEGF was more
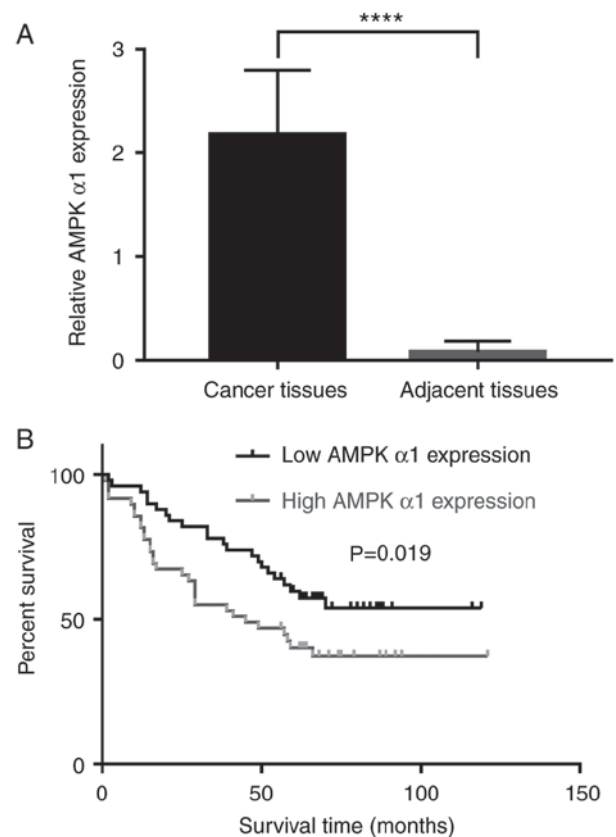

Figure 1. Expression level of AMPK α1 is upregulated in cancerous tissues and associated with poor prognosis. (A) Compared with that in non-tumor lung tissues, the expression level of AMPK α1 was significantly upregulated in cancer tissues. (B) Patients with high expression levels of AMPK α1 had a shorter overall survival compared with that of the low expression group. Data were expressed as the mean ± standard deviation. ****$P<0.0001$. AMPK α1, AMP-activated protein kinase α1.

highly expressed in NSCLC tissues compared with adjacent non-tumor lung tissues (Fig. 2A), and higher levels of VEGF were associated with a poorer prognosis (Fig. 2B).

Patients with NSCLC that express high levels of AMPK α1 and VEGF have a shorter OS. Based on the conclusion that AMPK α1 and VEGF are associated with NSCLC, patients that expressed high levels of AMPK α1 and VEGF (n=31; Table II) were selected and further classified. Patients that expressed high levels of AMPK α1 and VEGF had a significantly shorter OS compared with those that expressed low levels of AMPK α1 and VEGF, and those with all other expression level combinations (AMPK α1 low, VEGF low; AMPK α1 high, VEGF low; and AMPK α1 low, VEGF high; Fig. 3A and B).

AMPK α1 positively regulates VEGF expression in NSCLC in vitro. The potential regulatory association between AMPK α1 and VEGF in NSCLC was investigated. Western blotting was performed to detect the effects AMPK α1 expression level on that of VEGF. As illustrated in Fig. 4A, downregulation of AMPK α1 resulted in decreased VEGFA protein expression in A549 cells, and upregulation of AMPK α1 resulted in increased VEGFA protein expression (Fig. 4B). The results indicated that AMPK α1 positively regulates VEGF expression in NSCLC.
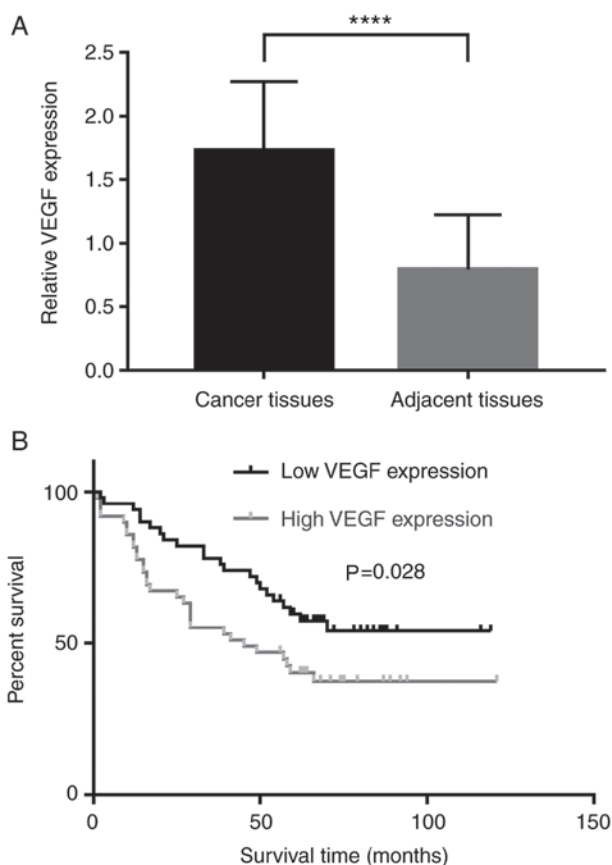
Figure 2. Increased expression of VEGF is associated with poor prognosis in non-small cell lung cancer. (A) Compared with that in non-tumor lung tissues, the expression level of VEGF was significantly upregulated in cancer tissues. (B) Patients with a high expression level of VEGF had a shorter overall survival compared with that of the low expression group. Data were expressed as the mean ± standard deviation. ****P<0.0001. VEGF, vascular endothelial growth factor.

Table II. Association between the expression levels of AMPK α1 and VEGF.

VEGF expression	AMPK α1 expression		P-value
	High	Low	
High	31	18	0.006
Low	18	32	

AMPK α1, AMP-activated protein kinase α1; VEGF, vascular endothelial growth.
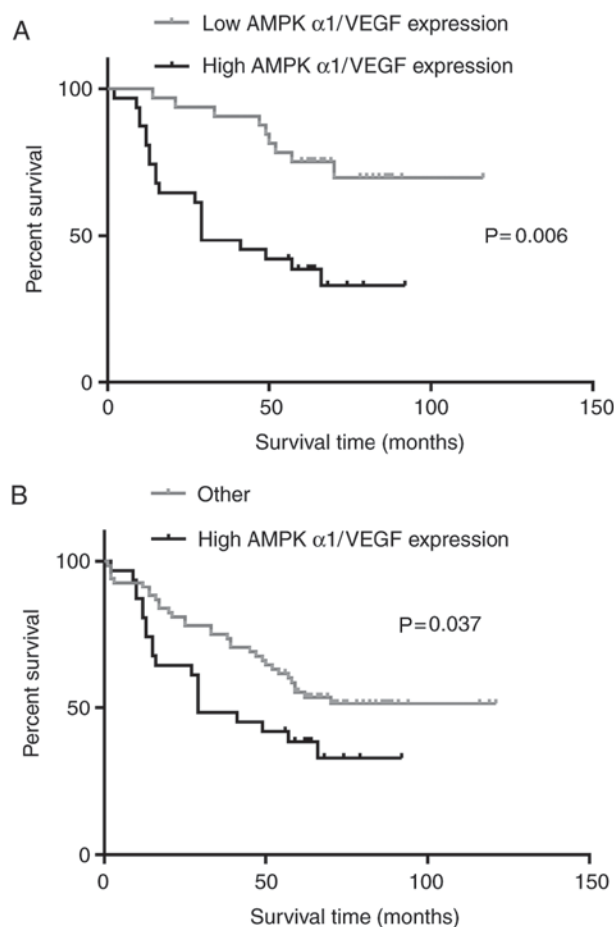

Figure 3. Patients with high expression of AMPK α1 and VEGF have shorter OS. (A) Compared with patients expressing low levels of AMPK α1 and VEGF, patients expressing high levels of these proteins exhibited a significantly shorter OS. (B) Compared with the remaining groups of patients (AMPK α1 low, VEGF low; AMPK α1 high, VEGF low; and AMPK α1 low, VEGF high), patients with NSCLC expressing high levels of AMPK α1 and VEGF displayed a significantly shorter OS. OS, overall survival; AMPK α1, AMP-activated protein kinase α1; VEGF, vascular endothelial growth factor; NSCLC, non-small cell lung cancer.

Discussion

Lung cancer is a commonly occurring malignancy with high mortality rates worldwide (11). Based on cell morphology, lung cancer is predominantly divided into two types: NSCLC and small cell lung cancer (SCLC), which account for 80 and 20% of all lung cancer cases, respectively. Although the detection rate and treatment have improved over time, the clinical outcomes of patients with lung cancer remain unsatisfactory (12,13). In addition, compared with SCLC, patients with NSCLC are less sensitive to chemotherapy. To achieve an improved outcome for patients with NSCLC, further studies to understand the mechanisms underlying tumorigenesis and metastasis, and the development effective treatments are required.

In the present study it was demonstrated that AMPK α1 was highly expressed in tumor tissues compared with non-tumor lung tissues, and overexpression of AMPK α1 was negatively associated with prognosis in patients with NSCLC. This observation of the aggressive role of AMPK α1 in NSCLC was consistent with previous findings (6,14). However, AMPK α1 has also been indicated as a tumor suppressor (15), and its deletion reportedly promotes cell proliferation and angiogenesis (16). This discrepancy in

different cancer types may be the result of different tissue systems, in addition to different targets and associated pathways. Furthermore, it was revealed that AMPK α1 positively regulated VEGF expression. Considering that VEGF is a well-known tumor driver gene, it was hypothesized that the
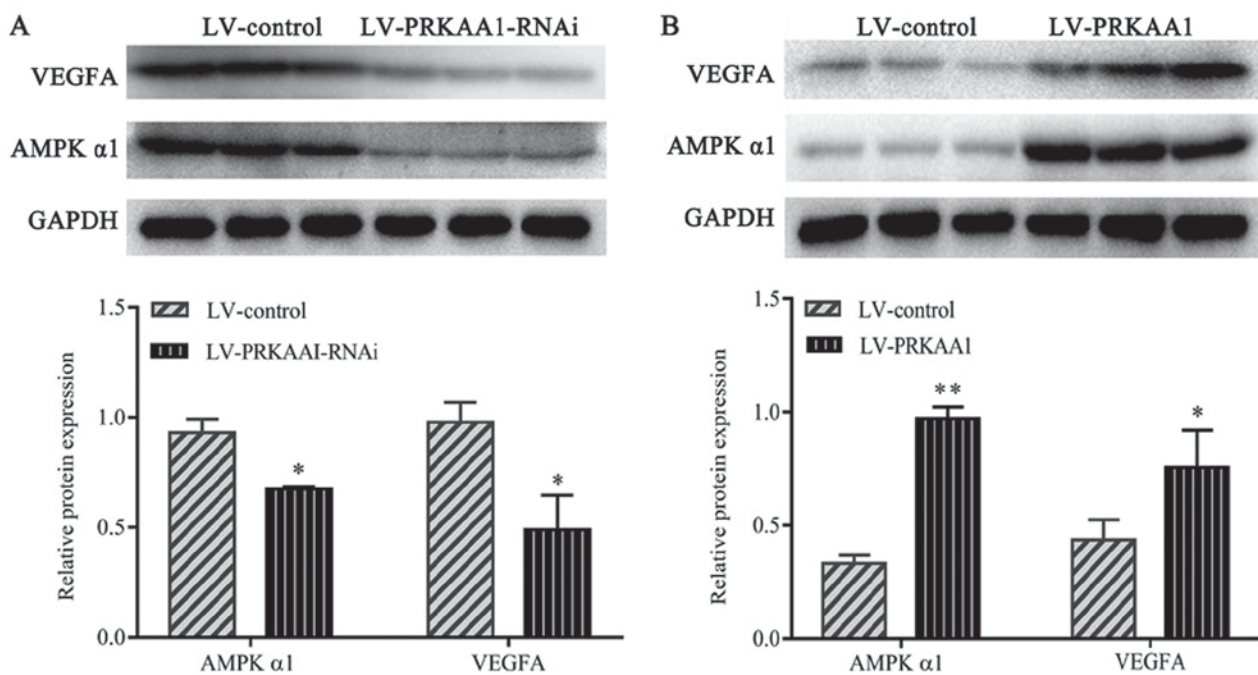
Figure 4. AMPK α1 positively regulates VEGF expression in NSCLC *in vitro*. (A) Downregulation of AMPK α1 decreased VEGFA protein expression levels in A549 cells. *P<0.05 vs. LV-control group. (B) Upregulation of AMPK α1 led to increased VEGFA protein expression levels. *P<0.05, **P<0.01 vs. LV-control group. LV-PRKAA1-RNAi, lentivirus expressing a short interfering RNA targeting *PRKAA1* (the gene encoding AMPK α1); LV-PRKAA1, lentivirus over-expressing *PRKAA1*. LV, lentivirus; AMPK α1, AMP-activated protein kinase α1; VEGF, vascular endothelial growth factor.

malignant role of AMPK α1 may be influenced by the regulation of VEGF expression in NSCLC. Anti-angiogenesis of cancer cells is a promising method to treat cancer. However to date, its efficacy has been limited (17). In the present study, a positive regulatory association was observed between AMPK α1 and VEGF, such that patients possessing tumors with high levels of these proteins had poorer clinical outcomes. Therefore, simultaneous targeting of AMPK α1 and VEGF may enhance the anti-cancer efficacy of anti-VEGF therapy.

The present study was limited in the following ways: Firstly, this was a retrospective, single institution study, and the number of specimens used to analyze the association between AMPK α1 and prognosis was small. Moreover, the exact mechanism of AMPK α1 regulation of VEGF in NSCLC was not investigated.

In conclusion, the present study identified an aggressive pro-cancerous effect of high levels of AMPK α1 in patients with NSCLC. A regulatory association between AMPK α1 and VEGF was also demonstrated. The results indicated that AMPK α1 is a potential biomarker and therapeutic target in NSCLC, which requires further investigation *in vitro* and *in vivo*.

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Availability of data and materials

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

Authors' contributions

XXX, HSW and MLF made contributions to conception and design of this study. WYX, CY, CBY, ZJ, GJJ and YJJ analyzed and interpreted the patients' data. GDH contributed to the conception and the design of this study and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Board of Subei People's Hospital (Yangzhou, China), and written informed consent was obtained from all participants.

Patient consent for publication

All of the patients provided written informed consent for the publication of any associated data.

Competing interests

The authors declare that they have no competing interests.

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