

Overexpression of C1QTNF6 in Esophageal Cancer and Promotes the Proliferation and Migration of Esophageal Cancer Cells

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Xia *et al.*: Effect of C1QTNF6 Biological Function on Esophageal Cancer

Esophageal cancer is a common cancer among people in the world. The molecular mechanism of complement C1q tumor necrosis factor related protein 6 in esophageal cancer is still unclear. This article aimed to clarify the role of complement C1q tumor necrosis factor related protein 6 in esophageal cancer. The expression of complement C1q tumor necrosis factor related protein 6 in esophageal cancer tissues was detected using quantitative real-time polymerase chain reaction and Western blot. Furthermore, short hairpin ribonucleic acid was used to knockdown the expression of complement C1q tumor necrosis factor related protein 6 in esophageal cancer cell lines. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide, wound healing assays and transwell assays were used to determine the role of complement C1q tumor necrosis factor related protein 6 in esophageal cancer cells. In our research, we found that the expression of complement C1q tumor necrosis factor related protein 6 was up-regulated in esophageal cancer tissues. The increased expression of complement C1q tumor necrosis factor related protein 6 was significantly correlated with tumor stage and lymph node metastasis. The proliferation and migration of esophageal cancer cells were inhibited after complement C1q tumor necrosis factor related protein 6-short hairpin ribonucleic acid transfection *in vitro*. These results suggested that complement C1q tumor necrosis factor related protein 6 serves as an oncogene and promotes cell proliferation and migration in esophageal cancer. In addition, complement C1q tumor necrosis factor related protein 6 may be a potential therapeutic target for esophageal cancer patients.

Key words: C1q tumor necrosis factor related protein 6, esophageal cancer, short hairpin ribonucleic acid, proliferation, migration, quantitative real-time polymerase chain reaction

Esophageal Cancer (EC) is the eighth most common cancer worldwide and has the sixth highest cancer mortality^[1-3]. Esophageal Squamous Cell Carcinoma (ESCC) has become the most frequent subtype of EC^[4,5]. Despite with the great advances in treatment, the 5 y survival rate of EC is remaining at 10 %-20 % related to proliferation and invasion of tumor cells^[6]. The molecular mechanisms of EC remain unclear to date. Thus, it is important for us to investigate the molecular mechanisms underlying EC tumorigenesis.

C1q is associated with the tumor necrosis factor superfamily in several biological processes, including inflammation, apoptosis and cell differentiation. C1q/Tumor Necrosis Factor-Associated Protein (CTRP protein) has been found to play a role in carcinogenesis and cancer progression^[7]. The C1q/TNF-related protein

family is consisted of 16 CTRP members. Among them, CTRP3, CTRP4 and Complement C1q Tumor Necrosis Factor Related Protein 6 (C1QTNF6) have been found to be associated with tumor deterioration. All CTRP members are secreted proteins that are widely expressed in various tissues and cell types^[8-12]. CTRP4 was shown to be a tumor-promoting inflammatory modulator and to promote tumor cell survival and reduce drug-induced apoptosis^[7]. These findings strongly suggest that CTRP4 is a potential therapeutic target. CTRP8 has been reported to be involved in brain cancer^[13]. It has also been shown to enhance motility and stromal invasion by glioblastoma cells^[14]. CTRP8-induced migration of human glioma cells revealed inhibition of small competitive peptides derived from C1QTNF6^[15]. Western blotting showed that C1QTNF6 was highly

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expressed in human hepatocellular carcinoma tissues. High expression of C1QTNF6 can activate Protein Kinase B (Akt) signaling pathway, increase tumor angiogenesis and reduce Human Liver Carcinoma Cells (HepG2) necrosis^[16]. C1QTNF6-interference inhibits Extracellular Signal-Regulated Kinase 1/2 (ERK1/2) signaling in mouse embryonic fibroblast cell line (3T3-L1) adipocytes^[17].

In our study, we found that C1QTNF6 was significantly higher in tumor compared to normal tissue. In addition, its biological effects on EC cancer cell lines were also identified. This article aimed to explore the relationship between C1QTNF6 and EC.

MATERIALS AND METHODS

Human tissue samples:

50 pairs of EC tissues and matched adjacent normal tissues were obtained from EC patients who underwent surgical resection at the First Affiliated Hospital of Soochow University from January 2017 to September 2020. All participants received no other treatment prior to surgery and signed written informed consent. Our study was approved by the Ethical Committee of the First Affiliated Hospital of Soochow University.

Cell culture:

Two EC cell lines i.e., Epithelial cell lines (TE-1) and human Esophageal Carcinoma cell lines (ECa-109) are typical representatives of esophageal adenocarcinoma and squamous cell carcinoma, so we used these two cell lines for our study. Two EC cell lines (TE-1 and ECa109) were incubated in Dulbecco's Modified Eagle Medium (DMEM) with 10 % fetal bovine serum (Thermo Fisher Scientific) at 37° in a 5 % Carbon dioxide (CO₂) atmosphere. After the confluence of cells reached 40 %-50 %, the cells would be used for transfection.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR):

Ribonucleic Acid (RNA) extraction from the cell lines and human tissues was performed with a TRIzol kit (Invitrogen, United States of America (USA)). The isolated RNA was measured at 260/280 nm using spectrophotometry (Thermo, San Jose, California, USA). All the RNA samples were stored at 80°. SYBR Premix Ex Taq II (Stratagene, USA) was used for qRT-PCR on ABI 7500 fast RT-PCR system (Applied, Biosystems). The primers were as follows. C1QTNF6-Forward: 5'-GAAAGGGTCTTTGTGAACCTTGA-3', C1QTNF6-Reverse, 5'-

CTGCGCGTACAGGATGACAG-3'; Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH)-Forward: 5'-TGACTTCAACAGCGACACCCA-3', GAPDH-Reverse, 5'-CACCTGTTGCTGTAGCCAAA-3'. GAPDH was amplified as an internal control. The relative gene expression was calculated by a 2^{-ΔΔCT} method.

Short hairpin Ribonucleic Acid (shRNA) establishment:

The siRNA is expressed instantaneously in the cell, while the shRNA is stabilized by virus-mediated transduction. In order, for the knockdown of C1QTNF6, a shRNA vector targeting the C1QTNF6 gene was established by Shanghai GeneChem Co., Ltd. The targeting sequences of C1QTNF6 were cloned into the lentiviral vector pGVX115-Green Fluorescent Protein (GFP) to obtain pGVX115-shC1QTNF6. The pGVX115-GFP was served as a control and was titled pGVX115-shCtrl.

Western blot:

Total protein was extracted with Radioimmunoprecipitation Assay (RIPA) buffer from the cultured cells and the protein concentration was detected by a Bicinchoninic Acid (BCA) protein assay kit (Bio-Rad, Italy). Proteins were isolated by 10 % Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), moved to Polyvinylidene Fluoride (PVDF) membranes and immunoblotted with the following primary antibodies: C1QTNF6 (1:500, SantaCruz, USA) and GAPDH (1:1000, SantaCruz, USA) overnight at 4°. After washing three times, the membranes were incubated with secondary antibody for 2 h at 37°. Protein bands were visualized using Enhanced Chemiluminescence (ECL) reagents (Pierce, Rockford, Illinois, USA).

Cell proliferation and wound healing assays:

Cell proliferation was measured using 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl-2H-Tetrazolium Bromide (MTT) assay. Cells were seeded in 96-well plates for 24 h. 20 μl of MTT solution (MedChemExpress, Monmouth Junction, New Jersey, USA) was added to each well at 1, 2, 3, 4 and 5 d after transfection. The absorbance values at 490 nm were detected using a microplate absorbance reader (MRXII, Dynex Technologies, Chantilly, Virginia, USA). Each experiment was performed in triplicate.

Cell migration was performed using wound healing assays. For wound healing assays, cells were plated into six-well plates with each experimental group.

A liner wound was made by scraping a non-opening Pasteur pipette across the confluent cell layer. Cells were washed twice to remove detached debris. Then, size of wounds was observed and measured at indicated times. Each experiment was performed in triplicate.

Transwell assay:

After transfection for 72 h, cells were inoculated into the 6-well transwell upper chamber with a density of 25 000 cells/well. The transwell assay was conducted based on the manufacturer's instructions. The transwell chamber was then placed in a humidified incubator with 5 % CO₂ at 37° for 48 h. The lower chamber was stained with hematoxylin and photographed.

Statistical analysis:

All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) 17.0 (IBM Corporation, Armonk, New York, USA). Data were expressed as mean±Standard Deviation (SD). Statistical differences were analyzed by Student's t-test or one-way Analysis of Variance (ANOVA), $p < 0.05$ was defined statistically significant.

RESULTS AND DISCUSSION

C1QTNF6 is up-regulated in EC tissues were shown in fig. 1. Western blot and qRT-PCR were used to detect the expression levels of C1QTNF6 in 50 paired EC tissues and adjacent normal tissues. With the increasing of Tumor (T) stage, C1QTNF6 expression level increased continuously ($p < 0.05$; fig. 1A). According to the

results of chi-square test, the expression of C1QTNF6 was significantly up-regulated in positive lymph node compared with negative lymph node ($p < 0.05$; fig. 1B). Then, we selected 10 of these patients and found higher levels of C1QTNF6 expression level than in adjacent tissues ($p < 0.05$; fig. 1C). To assess whether C1QTNF6 expression is related with the development of EC, the relationship between C1QTNF6 and clinicopathological characteristics of EC patients was conducted. As displayed in Table 1, we divided the patients into high-expression group and low-expression group according to the median value in EC tissues and we found C1QTNF6 expression was significantly correlated with differentiation ($p = 0.039$), lymph node metastasis ($p = 0.031$) and T stage ($p = 0.022$). However, no significant correlation was identified between the expression of C1QTNF6 and other characteristics such as sex, age and histology (all $p > 0.05$). Univariate and multivariate Cox analysis of clinical parameters associated with overall survival was shown in Table 2, $p < 0.05$.

Knockdown expression of C1QTNF6 by shRNA lentivirus transfection into TE-1 and Eca-109 cell lines was shown here. Endogenous C1QTNF6 expression in TE-1 and Eca-109 cell lines was detected by qRT-PCR. The assay showed that C1QTNF6 was largely expressed both in TE-1 and Eca-109 cell lines, showing that TE-1 and 177 Eca-109 were fit for knocking down analysis (fig. 1D).

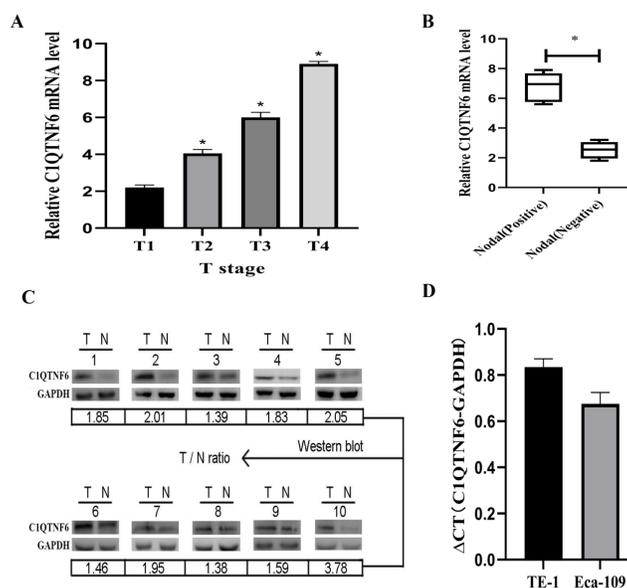


Fig. 1: The expression of C1QTNF6 in EC tissues and EC cell lines, * $p < 0.05$

TABLE 1: RELATIONSHIP BETWEEN C1QTNF6 AND CLINICOPATHOLOGICAL CHARACTERISTICS OF EC PATIENTS

Characteristics	All patients	C1QTNF6 low expression	C1QTNF6 high expression	P
n	50	24	26	
Age (y)				0.866
<60	22	10	12	
≥60	28	13	9	
Sex				0.897
Male	27	14	13	
Female	23	12	11	
Histology				0.772
Adenocarcinoma	7	3	4	
Squamous cell carcinoma	43	20	23	
Differentiation				0.039
Well+moderate	12	4	8	
Poor	38	14	24	
Lymph node metastasis				0.031
Positive	34	12	22	
Negative	16	5	11	
T stage				0.022
≤II	14	4	10	
>II	36	12	24	

TABLE 2: UNIVARIATE AND MULTIVARIATE COX ANALYSIS OF CLINICAL PARAMETERS ASSOCIATED WITH OVERALL SURVIVAL

Parameters	Univariate analysis			Multivariate analysis		
	HR	95 % CI	p	HR	95 % CI	p
C1QTNF6	3.528	1.849-5.114	0.012*	3.382	1.738-4.993	0.009*
Age (y)	1.721	0.664-2.804	0.201	1.806	0.773-2.975	0.197
Sex	1.016	0.464-1.682	0.593	1.031	0.453-1.691	0.709
Histology	2.173	0.559-3.975	0.404	2.022	0.528-3.593	0.516
Differentiation	1.773	0.491-2.921	1.076	1.742	0.515-3.041	1.011
Lymph node metastasis	1.128	0.516-1.783	0.825	1.298	0.583-1.884	0.818
T stage	0.936	0.482-1.577	0.116	0.885	0.407-1.764	0.183

Note: HR: Hazard Ratio; CI: Confidence Intervals, *p<0.05

In order to examine the function of C1QTNF6 in TE-1 and Eca-109 cell lines, lentiviruses carrying C1QTNF6-shRNA and shCtrl were used to infect TE-1 and Eca-109 cell lines. Transfection efficacy was examined by GFP (fig. 2A). The transfection efficiency was assessed by qRT-PCR and the results suggested that C1QTNF6 messenger RNA (mRNA) expression level was significantly reduced in the C1QTNF6-shRNA group compared with the shCtrl group (fig. 2B).

Knockdown of C1QTNF6 inhibited cell proliferation of TE-1 and Eca-109 cell lines *in vitro* was shown in fig. 3. To decipher the effect of C1QTNF6 on growth of EC cells, MTT assay was used. Cell proliferation of TE-1 cell lines following shC1QTNF6/shCtrl infection was detected by MTT assay from d 1 to d 5 was shown in fig. 3A. Cell proliferation of Eca-109 cell lines following shC1QTNF6/shCtrl infection was detected by MTT assay from d 1 to d 5 was shown in fig. 3B. As shown in fig. 3A and fig. 3B, compared with shCtrl group, the cell proliferation ability of C1QTNF6-shRNA group was significantly decreased between d 1 to d 5 in TE-1 and Eca-109 cell lines ($p < 0.05$).

Knockdown of C1QTNF6 gene expression in EC cells inhibiting proliferation *in vitro* was shown in fig. 4. Cell proliferation was also evaluated using wound healing assays. Cell distance of TE-1 cell lines following shC1QTNF6/shCtrl infection was detected

by wound healing assay from 0 h to 48 h was shown in fig. 4A. Cell distance of Eca-109 cell lines following shC1QTNF6/shCtrl infection was detected by wound healing assay from 0 h to 48 hours was shown in fig. 4B. As demonstrated in fig. 4A and fig. 4B, compared with the shCtrl group, the distance between cells was significantly increased after C1QTNF6-shRNA transfection into TE-1 and Eca-109 cell lines ($p < 0.05$).

Knockdown of C1QTNF6 inhibited cell migration of TE-1 and Eca-109 cell lines *in vitro* was shown in fig. 5. Cell migration of TE-1 cell lines following shC1QTNF6/shCtrl infection was detected by transwell assay after 72 h was shown in fig. 5A. Cell migration of Eca-109 cell lines following shC1QTNF6/shCtrl infection was detected by transwell assay after 72 h was shown in fig. 5B. Cell counts of TE-1 and Eca-109 cell lines following shC1QTNF6/shCtrl infection were investigated by transwell assay after 72 h was shown in fig. 5C. As displayed in fig. 5A-fig. 5C, compared with the shCtrl group, the cell counts of cell migration was significantly increased after C1QTNF6-shRNA transfection into TE-1 and Eca-109 cell lines ($p < 0.05$).

EC occurrence is a complex process, including vascular production, cell adhesion, motility, Extracellular Matrix (ECM) degradation and angiogenesis^[18-22]. EC patients diagnosed at an advanced stage usually have a poor prognosis^[6]. Therefore, to improve the clinical outcomes of EC patients, it is crucial for us to identify

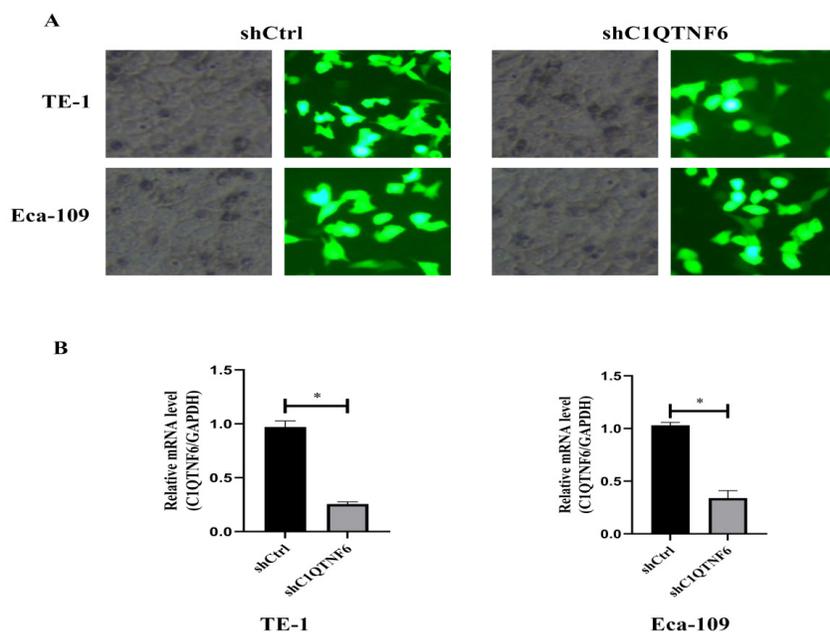


Fig. 2: Expression level of C1QTNF6 in TE-1 and Eca109 cell lines was examined by qRT-PCR following shC1QTNF6/shCtrl transfection, * $p < 0.05$

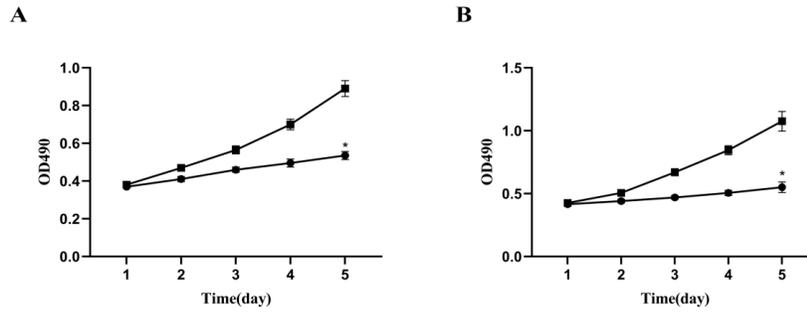


Fig. 3: Knockdown C1QTNF6 gene expression in EC cells inhibiting proliferation *in vitro*, (◐) shCtrl and (◑) shC1QTNF6; *p<0.05

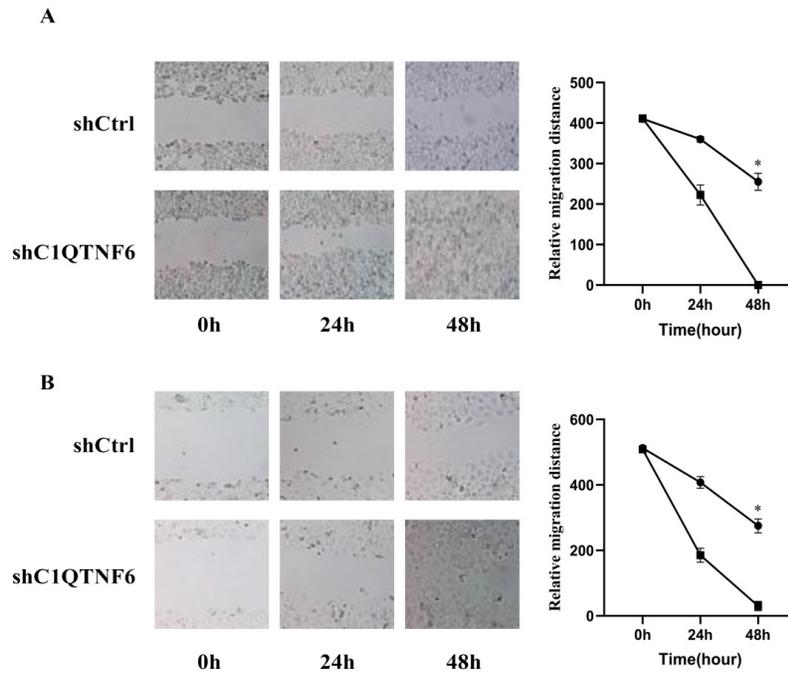


Fig. 4: Knockdown C1QTNF6 gene expression in EC cells inhibiting proliferation *in vitro*, (◐) shCtrl and (◑) shC1QTNF6; *p<0.05

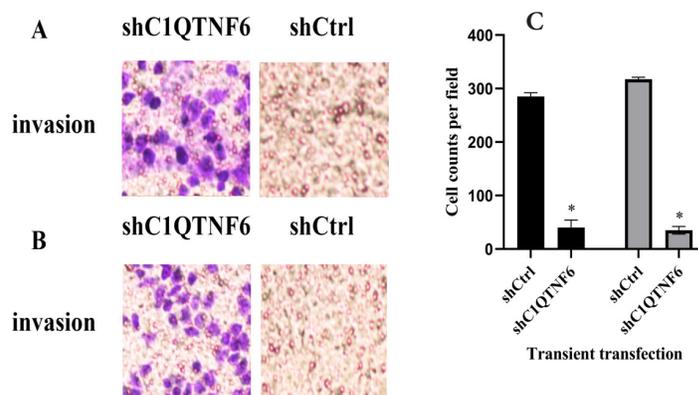


Fig. 5: Knockdown of C1QTNF6 gene expression in EC cells inhibiting migration *in vitro*, (◐) TE-1 and (◑) Eca-109; *p<0.05

novel biomarkers. With the development of technique, sequencing analysis has been applied to understand its molecular mechanisms comprehensively^[19]. In our research, 50 pairs of EC tumors and adjacent non-cancer tissues were conducted with RNA sequencing and we discovered that C1QTNF6 is differentially expressed. At present, C1QTNF6 up-regulation was found in gastric cancer^[23]. Previous studies have confirmed that down-regulation of C1QTNF6 mRNA promoted the invasiveness of human breast cancer cell line (Michigan Cancer Foundation-7 (MCF-7)). In the above studies, C1QTNF6 was found to be directly targeted by microRNA-29b (miR-29b), which may be regarded as a tumor suppressor or promoter, depending on the cell or tissue type. However, little is known about the function of C1QTNF6 in EC. The goal of this article was to search the biological role of C1QTNF6 in EC cells and to clarify its function in the occurrence of EC.

In this study, C1QTNF6 was significantly up regulated in EC tissues. Moreover, up-regulation of C1QTNF6 was significantly correlated with differentiation, lymph node metastasis and T stage, which suggested that C1QTNF6 may be correlated with the prognosis of EC patients and may be related with the progression of EC. In addition, C1QTNF6 was largely expressed in TE-1 and Eca-109 cell lines, C1QTNF6 shRNA could be constructed and transfected into TE-1 and Eca-109 cell lines.

In conclusion, these results displayed that down-regulation of C1QTNF6 expression, which could inhibit cell proliferation and migration ability *in vitro*. However, there are some limitations in our study. The sample size is restricted, which may influence the results. Therefore, more experiments are needed to elucidate the specific molecular mechanism of C1QTNF6 in EC in the future. Nevertheless, we should conduct more *in vivo* experiments to validate the oncogene effects of C1QTNF6. As a result, this article indicates that C1QTNF6 has significant biological implications in EC. Therefore, C1QTNF6 might serve as an oncogenic role in EC and be a potential target for treatment of EC.

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Haitao Ma designed this study. Haifeng Xia, Fei Lu, Xiaojun Yu and Yu Feng performed experiments and analyzed data. This paper was written by Haifeng Xia.

Conflict of interests:

The authors declared no conflict of interest.

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