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Glutamine metabolism in the proliferation of GS-expression pituitary tumor cells

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Abstract

Objective: Many cancer cells cannot survive without exogenous glutamine (Gln); however, cancer cells expressing glutamine synthetase (GS) do not have this restriction. Previous metabolomics studies have indicated that glutamine metabolism is altered during pituitary tumorigenesis. However, the main role of Gln in pituitary adenoma (PA) pathophysiology remains unknown. The aim of this study was to evaluate the expression of GS and the main role of Gln in human PAs.

Methods: We used cell proliferation assay and flow cytometry to assess the effect of Gln depletion on three different pituitary cell lines and human primary PA cells. We then investigated the expression level of Gln synthetase (GS) in 24 human PA samples. At last, we used LC-MS/MS to identify the differences in metabolites of PA cells after the blockage of both endogenous and exogenous Gln.

Results: PA cell lines showed different sensitivities to Gln starvation, and the sensitivity is correlated with GS expression level. GS expressed in 21 out of the 24 human PA samples. Furthermore, a positive p53 and ki-67 index was correlated with a higher GS expression level (P < 0.05). Removal of both endogenous and exogenous Gln from GS-expressing PA cells resulted in blockage of nucleotide metabolism and cell cycle arrest.

Conclusions: Our data indicate that GS is needed for PA cells to undergo proliferation during Gln deprivation, and most human PA cells express GS and might have a negative response to exogenous Gln depletion. Moreover, Gln is mainly responsible for nucleotide metabolism in the proliferation of GS-expressing pituitary tumor cells.

Background

Pituitary adenoma (PA) is the second most common brain tumor. Except for prolactinoma, pituitary tumors associated with acromegaly (growth hormone-secreting) and Cushing’s disease (adrenocorticotropic hormone-secreting) as well as clinical nonfunctioning pituitary adenomas (NFPAs) are generally treated with transsphenoidal surgery as the first-line therapy. However, since some PAs invade the cavernous sinus or surrounding bone in the sellar region, it is sometimes difficult to achieve total surgical resection (1). Moreover, the currently available pharmacotherapies are not satisfactory. Thus, there is a need to explore novel approaches for treating PA.

Metabolic abnormalities, including the Warburg effect and glutamine (Gln) addiction, have been considered as a hallmark of cancer cells (2). The Warburg effect refers to the observation that cancer cells tend to favor anaerobic glycolysis even when oxygen is abundant. Despite PA being a benign tumor, we previously reported that lactate dehydrogenase A (LDHA), a key glycolysis enzyme, was involved in promoting its progression. Therefore, LDHA might be a promising therapeutic target for treating PA (3).

Gln is the most abundant amino acid in the human body and is a non-essential amino acid in the
physiological state as it is endogenously synthesized. However, most cancer cells die when they lack exogenous Gln, a phenomenon termed as ‘Gln addiction’ (4). Gln constitutes a major metabolic fuel for some types of tumor cells and is a precursor for various biological macromolecules, such as amino acids, proteins, lipid, and nucleotides. Therefore, it is not surprising that Gln depletion induces severe metabolic stress that may lead cells to undergo apoptosis (5, 6). Therapeutic agents targeting Gln transporters or glutaminase have been shown to be effective (7, 8, 9, 10).

However, not all cancer types rely on exogenous Gln to survive, such as those that express Gln synthetase (GS). The gene GLUL codes for GS, which is an enzyme that catalyzes Gln synthesis from glutamate (Glu) and NH4+. The presence or absence of GS expression is an important survival factor after exogenous Gln depletion. Therefore, cancers such as luminal breast cancer (11), MET-induced liver cancer (12), glioma (13), and low invasive ovarian cancer (14) are insensitive to Gln deprivation owing to the high GS expression.

The glutamine- or glutamate-related pathways have also been reported to be altered in pituitary gland tumorigenesis using mass spectrometry or NMR spectrometry. Rahmi et al. compared blood samples from the bilateral inferior petrosal sinus in corticotropinoma patients and healthy controls. They found that Glu metabolism was the most affected metabolic pathway (15); however, they did not show how the Glu metabolism pathway was affected. Similarly, Oklu et al. also observed that Glu and Gln were up-regulated in prolactinoma tissues compared with gonadotropin-secreting PAs (16). These data indicate that Glu/Gln metabolism is altered in pituitary tumorigenesis and that glutamine/glutamate metabolism processes may differ for the different clinical types of PA. However, the specific role of Gln in the pathophysiology of PA is still unknown. Hence, in this study, we aimed to determine the main role of Gln in human PAs as well as GS expression.

Materials and methods
Cell culture and transfection
Rat GH3 (ATCC, CCL-82.1) and MMQ (ATCC, CRL-10609) cells as well as mouse AtT-20 (ATCC, CCL-89) PA cells were all cultured in Ham’s F-12K medium (GIBCO, 21127-022, with 2 mM Gln) containing 2.5% fetal bovine serum (GIBCO, 10100-147) and 15% horse serum (GIBCO, 16050-122) in a humidified atmosphere of 5% CO2 at 37°C. For Gln-free cell culture and certain experiments, custom-made Gln-free Ham’s F-12K medium (GIBCO, with 0 mM Gln) was used to dialyze fetal bovine serum and horse serum. Thereafter, 2.5% dialyzed fetal bovine serum and 15% horse serum were added to Gln-free Ham’s F-12K medium.

Cells were transfected with the negative control or GS siRNA (Gema, Shanghai, China) using Lipofectamine 2000 (Invitrogen Life Technologies, 11668-027) according to the manufacturer’s instructions. The GS siRNA sequence was ‘GCATCAAGCAGATGTACAT’.

Cell proliferation assay
We used the WST-8 Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Mashiki-machi, Kumamoto, Japan) to measure cell proliferation based on the manufacturer’s instructions. The doubling time (DT) was obtained using the exponential growth equation with GraphPad Prism 6.0. The percentage of growth inhibition was calculated according to the following equation: (1 − (DT + Gln)/(DT − Gln)) × 100.

 Colony-forming assay
GH3 cells were plated at 200 cells/well in 6-well plates in complete F-12K, in the absence or presence of Gln or 1 U/mL L-asparaginase (Sigma, A3809) and 1 mM L-methionine sulfoximine (Sigma, M5379) as indicated. After 14 days, cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Visible colonies were manually counted.

Patients
We obtained 24 human pituitary tumor samples (five somatotroph adenomas, five prolactinomas, five corticotropinomas, and nine nonfunctional adenomas) in patients who underwent transphenoidal surgery at the multidisciplinary center for pituitary adenomas in the Chongqing of the Xinqiao Hospital. Individual tumors were diagnosed according to clinical and endocrine assessments with additional information obtained by pathological evaluation. Tumor invasion was determined based on a preoperative radiological investigation using Knops’s classification (14) combined with intraoperative findings. Based on the Knops’s classification system, noninvasive adenomas were grade 0–2 and invasive adenomas were grades 3–4. Clinical data regarding sex,
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Immunohistochemistry

Tumor tissue was fixed in 4% paraformaldehyde for 24 h and embedded in paraffin. Embedded tumor tissues were sectioned at 5 µm for subsequent immunohistochemistry (IHC) staining. IHC staining was performed according to standard protocols using Anti-GS (1:50) antibodies.

The immunoreactivity (IR) of GS was evaluated as previously reported (17, 18). We used a Leica DM IRB microscope with a square grid inserted into the eyepiece to examine an area of 781.250 µm² (200 high power nonoverlapping fields with widths of 0.0625 × 0.0625 mm). The staining intensity was assessed using a semiquantitative three-point scale within the scope of the IR and was defined as absent (−, 1), weak (+, 2), moderate (+++, 3), or strong (+++, 4). These scores represented the predominant staining intensity in each section and were calculated as an average of the selected samples.

Primary culture of human PA cells

We obtained six primary human PA cells from harvested human PA specimens from patients undergoing transphenoidal surgery at the Xinqiao Hospital in Chongqing, China (Supplementary Table 2). Fresh tissues were washed with 1× PBS and minced into small pieces. Tissue fragments were then digested with type I collagenase for 2 hours at 37°C. An equal volume of MEM (GIBCO, 11095-080) supplemented with 10% fetal bovine serum was added, and the cell suspension was filtered through a 200-µm filter. After two washes with PBS, the cells were resuspended in complete MEM and cultured in a humidified atmosphere of 5% CO₂ at 37°C.

Western blot analysis

We used antibodies against the following proteins: GS (Abcam, ab176562), β-tubulin (Abcam, ab75991), Cyclin D1 (Cell Signaling Technology, 2978), Cyclin A2 (Beyotime, Shanghai, China, AF2524), and Cyclin E1 (Beyotime, AF2491).

Cell extracts equal to 40 µg protein were subjected to SDS-PAGE and then transferred onto polyvinylidene difluoride membranes. The membranes were blocked for 2 h at room temperature with 5% skimmed milk in Tris-buffered saline containing 0.05% Tween 20, incubated with rabbit antibodies against rat GS (1:1000), β-tubulin (1:10,000), Cyclin D1 (1:1000), Cyclin A2 (1:1000), and Cyclin E1 (1:1000).

Flow cytometry

For cell cycle analysis, cells were collected at the indicated time points for the relevant treatments and fixed in 70% ethanol at 4°C overnight. Cells were resuspended and stained in 1 mL PBS containing 50 µg/mL PI (BD Biosciences Pharmingen, 556463) and then analyzed using flow cytometry (FACScoule; BD Biosciences Pharmingen). For cell apoptosis analysis, cells were collected at the indicated time points for the relevant treatments. Apoptosis was assessed using a FITC-Annexin V Apoptosis Detection Kit (BD Biosciences Pharmingen, 556547) based on the manufacturer’s instructions, and cells were detected using flow cytometry.

Liquid chromatograph mass spectrometer (LC-MS) analysis for metabolites

Cells were seeded in 10-cm dishes. After 24 h growth, the medium was substituted with fresh medium in the presence (2 mM) or absence of Gln combined with MSO. After the stipulated experimental times, cells were washed with ice-cold PBS, and metabolites were extracted using 1 mL cold methanol acetonitrile-water solution (v/v, 2:2:1). Ultrasonic crushing was performed at low temperature, and the protein was incubated at −20°C for 1 h for precipitation at 13,000 g. The protein was then centrifuged at 4°C for 15 min, and the supernatant was removed, freeze-dried, and stored at −80°C for later use. Next, we added 100 µL acetonitrile for mass spectrometry analysis. The samples were re-dissolved in an aqueous solution (acetonitrile:water=1:1, v/v), vortexed, centrifuged at 14,000 g at 4°C for 15 min, and then the liquid supernatant was removed for analysis. We subsequently separated the obtained samples using Agilent 1260 HPLC system. Agilent 6460 QqQ mass spectrometer (Agilent Technologies) was used and mass spectrometry analysis was performed as previously described (19).

Statistical analysis

The data were expressed as mean±S.E.M. The correlations between the GS levels and PA clinical characteristics were determined using the chi-square test. Additionally, we used the two-tailed Student’s t-test to determine the statistical significance of between-group differences.
Data analyses were conducted using SPSS for Windows version 13.0 (SPSS Inc). P values less than 0.05 were considered statistically significant.

Results

PA cell lines showed different sensitivities to Gln starvation

To explore the response of PA cell lines to Gln starvation, we used Gln lacking F-12K medium, and the serum was dialyzed to remove Gln. Compared with the normal control, Gln withdrawal showed no significant effect on proliferation of GH3 cells; however, it inhibited the proliferation of MMQ and AtT20 cells at 64% and 20%, respectively (Fig. 1A and B). Flow cytometric apoptosis assay revealed that Gln withdrawal induced apoptosis in MMQ cells but had no significant effect on GH3 and AtT20 cells (Fig. 1C).

We also used L-asparaginase (ASNase), which can simultaneously decompose both asparagine (Asn) and Gln, and found that the proportion of cells that it inhibited was similar to the proportion of cells inhibited in a Gln-lacking medium (Fig. 1D). These results indicated that the response of PA cells to exogenous Gln starvation is heterogeneous.

Sensitivity to Gln starvation is correlated with GS expression level

GS localizes in the cytoplasm and catalyzes Gln synthesis endogenously. To determine the mechanism underlying the heterogeneity of the response of PA cells to exogenous Gln starvation, we first assessed the expression of GS in GH3, MMQ, and AtT20 cell lines. As expected, GS was significantly higher expressed in GH3 cells compared to AtT20 or MMQ cells (P<0.001). Furthermore, Gln starvation significantly increased GS expression in GH3 (P<0.001) and AtT20 (P<0.05) cells but showed no significant effect on MMQ cells (Fig. 2A). Then we tested primary cells derived from three human NFPA patients (case 1–3) and three human growth hormone (GH) secreting PA patients (case 4–6). Five out of the six patients’ primary cells also significantly increased GS expression levels under Gln starvation (Fig. 2B).

Next, we used GS-siRNA to perform a knockdown of GS expression in GH3 cells. Compared with controls, GS knockdown cells were more sensitive to exogenous Gln starvation, but no significant effect was observed in the presence of exogenous Gln (Fig. 2C). L-methionine sulfoximine (MSO), an irreversible inhibitor of GS, also sensitized GH3 cells to Gln starvation. MSO alone also had no effect on cell proliferation in the presence of exogenous Gln. A combination of ASNase and MSO significantly suppressed the proliferation of all PA cell lines (Fig. 2E). The colony formation assay also yielded similar results (Fig. 2F and G). Furthermore, Asn depletion alone had no effect on GH3 cell proliferation, and Asn supplementation could not rescue GH3 cell proliferation under Gln starvation (Fig. 2D). These data indicate that GS expression determines the sensitivity of PA cells to exogenous Gln depletion.

GS expression in human PA tissues

Since GH3, MMQ, and AtT20 cells secrete GH, prolactin (PRL), and adrenocorticotropic hormone (ACTH), respectively, we sought to determine whether GS was differentially expressed among the various types of human hormone-secreting PAs. Thus, we analyzed GS expression

Figure 1

PA cell lines showed different sensitivity to Gln starvation. (A) GH3, MMQ, and AtT20 cell proliferation with/without Gln were tested by the CCK-8 assay (n = 5, Mean ± S.E.M.). (B) Growth inhibition caused by Gln starvation. (C) Apoptosis of cell lines incubated for 48 h with/without Gln. (n = 3, Mean ± S.E.M.). (D) Cells were incubated for 48 h in medium with/without L-asparaginase (ASNase). OD values were tested by CCK-8 assay (n = 5, Mean ± S.E.M.). Gln (+): containing 2 mM Gln; Gln (−): containing 0 mM Gln; ns: no significant. ***P < 0.001.
of 24 human PA tissues using IHC. In total, four hormone types of PAs were included: five GH-secreting PAs, five ACTH-secreting PAs, five PRL-secreting PAs, and nine NFPAs. We divided the observed expression into four grades from low to high (score 1–4) (Fig. 3A). Results from IHC staining indicated that GS was expressed in the vast majority (21/24) of patients and was only absent in three patients (Fig. 3B). We further analyzed the relationship between GS expression level and patients’ age, sex, tumor size, hormone type, cavernous sinus invasion, and ki-67 index (Table 1). The results showed that a positive ki-67 index (ki-67 >3%) and positive p53 both correlated with a higher GS expression (P<0.05) (Fig. 3C and D). Comparison among the four hormone types showed that GH-secreting PAs had the highest GS expression level, consistent with that observed in the cell lines (Fig. 3E).

Gln deprivation induced cell cycle arrest but not cell death in GS-expressing PA cell

Previous IHC results have indicated that most human PAs expressed GS. Among the three cell lines, GH3 cells...
represented the most significant GS expression (Fig. 2A), therefore, we used GH3 cells to further explore the main function of Gln in PA cells. GH3 cells were still in the adherent state after 3 days of both endogenous and exogenous Gln deprivation (Fig. 4A). In addition, GH3 cell proliferation was rescued after Gln addition to the medium after 3 days of exogenous and endogenous Gln deprivation (Fig. 4A and B). Furthermore, the CCK-8 assay revealed that exogenous and endogenous Gln deprivation did not influence cell survival in five of six human primary PA cells (Supplementary Fig. 1). These phenomena indicated that exogenous and endogenous Gln deprivation only inhibited the cell cycle but did not induce cell death. To confirm this hypothesis, we analyzed the cell cycle distributions and apoptosis using Annexin V flow cytometry. We observed no significant difference in the percentage of apoptotic cells (Fig. 4C). However, the cell cycle distribution showed that Gln deprivation arrested cell cycle progression at the S phase (Fig. 4D). EDU-labeled cells were also remarkably reduced in the Gln deprivation group (Fig. 4E). Cyclins are a family of proteins containing the conserved cyclin box, which mediate binding to cyclin-dependent kinases and are involved in cell cycle control. Cyclin D controls the G1 phase whereas cyclin A and cyclin E drives G1/S phase transition (20). A combination of ASNase and MSO significantly decreased cyclin A, cyclin D1, and cyclin E levels after 48 h (Fig. 4F).

**Gln deprivation affected nucleotide metabolism in GS-expressing PA cell**

To further determine the key metabolic role of Gln in GS-expressing cells, we used LC-MS/MS to identify the
differences in metabolites. The most changed metabolites are shown in the heat map (Fig. 5A). We observed that the levels of tricarboxylic acid (TCA) cycle intermediates derived from Gln anaplerosis, such as α-KG and its downstream metabolites succinate and fumarate, remain unchanged under both endogenous and exogenous Gln deprivation (Fig. 5B). However, as observed for dCTP, dCMP, deoxyguanosine, and deoxycytidine, we found a significant decrease in the intracellular level of inosine monophosphate (IMP), the key metabolite of the nucleotide de novo synthesis pathway, under Gln deprivation (Fig. 5C), indicating a blockage of the nucleotide de novo synthesis pathway. Conversely, we observed a significant increase in the intracellular levels of inosine, guanosine, cytidine, and uridine (Supplementary Fig. 2), indicating a blockage of the nucleotide salvage pathway. Pathway enrichment of the changed metabolites also indicated significant changes in the purine and pyrimidine metabolic pathways (Supplementary Fig. 3).

Our previous results have confirmed that the S phase of cell cycle was blocked when glutamine was depleted (Fig. 4D and F). DNA replicates among S phase, and this process need a lot of nucleotides. We checked the levels of the deoxynucleotides in our data, which were the direct synthetic material of DNA. As expected, we found that all the detected deoxynucleotides were decreased, including dCTP, dCMP, deoxyguanosine, and deoxycytidine. We then tried to use deoxynucleotides to rescue cell proliferation. The results showed that dCTP and dUTP could partly rescue GH3 cell proliferation in W condition. Besides, L-glutamine is a coding amino acid in protein synthesis. However, we found that cycloheximide (CHX), an eukaryote protein synthesis inhibitor (4), did not affect the rescue effect of dCTP and dUTP (Supplementary Fig. 4A), suggesting that protein synthesis blockage is not the main reason for the suppression of cell proliferation. Furthermore, we noticed that after Gln deprivation for 72 h, the DNA concentration of GH3 cells significantly
increased after readdition of glutamine (Supplementary Fig. 4B). Together, the results indicate that glutamine is mainly used to support nucleotides synthesis in the proliferation of GS-expressing pituitary tumor cells.

**Discussion**

The renewed attention to the metabolic characteristics of cancer cells and the recognition that changes in cell metabolism are a critical hallmark of cancer that have stimulated the exploration of targeting the metabolic specificity of cancer cells as a new therapeutic strategy. The important role of Gln has been confirmed in many cancers; however, little attention has been paid to the potential role of Gln metabolism in the progression of PA. To the best of our knowledge, this is the first study to demonstrate the effects of Gln depletion on PA cells. We show that Gln depletion may lead to increased GS expression in most PA cells, and the sensitivity to Gln depletion depends on the presence or absence of GS expression. We also observed GS expression for most human PA samples and that the positive p53 and ki-67 index was correlated with higher GS expression.
The proliferation of GS-expressing cell lines was completely suppressed after application of a combination of ASNase (a glutamine scavenger) and MSO (an irreversible GS inhibitor) due to blocking of nucleotide metabolism and cell cycle arrest.

Gln depletion induced apoptosis and suppressed cell growth in MMQ cells but had no significant effect on GH3 and AtT20 cells. These data indicate heterogeneity in the endogenous glutamine synthesis among different tumor types. The differences in the GS expression levels, especially the degree of compensatory enhancement after Gln depletion, explained this heterogeneity well. GS expression level has been shown to be negatively correlated with Gln depletion sensitivity in many cancer types (11, 12, 14). Pharmacological or siRNA-mediated GS inhibition significantly suppressed proliferation of glutamine-deprived cells but had no effect on cells grown under normal culture conditions. Taken together, these data indicate that GS is needed for PA cells to undergo proliferation during Gln deprivation.

To determine the GS expression levels in human PAs, we analyzed 24 human PA samples using IHC. The results indicated a correlation between a high ki-67 index and a high GS expression level. High GS expression has been reported to correlate with high aggressiveness and worse survival in glioblastoma (13, 21). We also noted that the GH subtype had the highest GS expression level among the four main subtypes of PA, which was consistent with the result that GH3, a GH-secreting cell line, had the highest GS expression level among the three cell lines. However, different to PRL-secreting cell line MMQ, human prolactinomas did not show a very low GS expression level. One possible explanation is that most prolactinomas were treated using drugs instead of surgery, and so the data of prolactinomas included in the present study did not represent the commonly observed prolactinomas. More importantly, we observed that irrespective of expression level, most PAs expressed GS, indicating that the majority of PAs might be insensitive to Gln depletion. Further, we tested and confirmed this phenomenon in five out of six primary PA cells. Despite cancer cells themselves, stromal-epithelial cells or astrocytes may express high levels of GS and synthesize Gln to support cancer cells regardless of whether they express GS (13, 22). Therefore, it may be necessary to include a GS inhibitor. However, MSO, which is the best-characterized GS inhibitor, has been largely avoided as a clinical cancer therapeutic agent due to CNS toxicity (23). Further examination of strategies to limit CNS toxicity by MSO and GS inhibition are needed, such as combination therapy, to lower the necessary dose or tumor-specific drug delivery. One such strategy involving the combination of MSO and L-asparaginase showed antitumor activity in a preclinical hepatocellular carcinoma xenografts model (24).

Gln involved in multiple metabolic pathways supports cell bioenergetics and biosynthesis. In several human cancer models, Gln has been shown to sustain cell growth through anaplerosis (25, 26, 27). Furthermore, Gln is also involved in many other pathways. In glioma cells, Gln fuels de novo purine biosynthesis rather than anaplerosis (13). However, Gln is necessary for sarcoma cells to support both optimal mitochondrial bioenergetics and nucleotide synthesis (28). Additionally, exogenous Gln is needed in oligodendroglioma cells for cell proliferation independent of anaplerosis or glutathione biosynthesis (29). We found that simultaneously blocking endogenous and exogenous Gln did not kill GH3 cells and that replenishing exogenous Gln could still restart their proliferation after Gln starving for 72 h. These phenomena indicated that Gln deprivation arrested the cell cycle, but did not induce cell death, which was confirmed by flow cytometry and EDU. Metabolomics analysis and replenishment results added to the evidence that the main role of Gln in the proliferation of GS-expressing cells is nucleotide metabolism rather than anaplerosis or protein translation.

However, the number of human PA samples in the present study is low and in vivo tests are lacking. Therefore, further clinical studies are needed to verify the present results and for further discovery.

Conclusions
In summary, the present study indicated that PA cells require GS to survive during Gln starvation. The majority of human PAs expressed GS, and positive p53 and ki-67 index correlated with higher GS expression. Blocking both endogenous and exogenous Gln in GS-expressing cells induced nucleotide metabolism disturbance and cell cycle arrest. Our results further the understanding of Gln metabolism in PA cells and may provide information for future clinical studies to develop novel therapeutic agents.

Supplementary materials
This is linked to the online version of the paper at https://doi.org/10.1530/EC-19-0515.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.
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Ethics and consent to participate
This study was approved by the Ethics and Clinical Research Committee of Xinqiao Hospital (China). Registration number: AF/SC-08/1.0. All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. We added this paragraph to 'Ethics and consent to participate' in the revised manuscript.

Consent for publication
Informed consent was obtained from all patients or their relatives.

Availability of data and materials
All data generated or analyzed during this study are included in this published article and its supplementary information files.

Author contribution statement
J H and S L conceived and designed the research and drafted the manuscript. J H, Q C, X D and X Z performed the experiments, analysis, and interpretation of data. X T and J H evaluated the IHC score. H Y critically reviewed and edited the manuscript. All authors have read and approved the final manuscript.

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