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LOSS OF *SETD2* IMPAIRS TIGHT JUNCTIONS AND PROMOTES THE TUMORIGENESIS OF GASTRIC CANCER

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ABSTRACT – *Objective:* Gastric cancer (GC) is the fourth leading cause of cancer-related deaths worldwide, and *MYC* overexpression has been observed in more than 40% of GC cases. Tight junctions (TJ) are intercellular junctions located at the most apical region of cell-cell contacts, and destruction of TJ can promote tumor metastasis. *SETD2* is a well-known trimethyltransferase that catalyzes the formation of the H3K36me3 modification. Loss of *SETD2* impairs gastric epithelial cells, promotes intestinal metamorphosis of gastric epithelial cells, and accelerates the migration and proliferation of GC cells. The purpose of this study is to explore the effect of *SETD2* loss on TJ and GC.

Materials and Methods: In this study, utilizing specimens from stomach-specific oncogenic *MYC* overexpression and *SETD2* deficiency mouse model (AMS), the author investigates the relationship between *SETD2* and TJ in GC.

Results: The results demonstrate that loss of *SETD2* downregulates the expression of TJ-associated genes (*MAPK10*, *ACTR3B*, and *CLDN24*), thereby impairing the expression of TJ-associated proteins (Claudin-1, Claudin-2, and ZO-1). Moreover, *SETD2* deficiency enhances cell viability and proliferation capacity in GC cells.

Conclusions: The loss of *SETD2* can disrupt the expression of TJ-related proteins and promote the tumorigenesis of gastric cancer. These findings shed light on the mechanism of tumorigenesis underlying GC and provide further theoretical support for the future development of targeted therapy against *SETD2* loss in GC patients.

KEYWORDS: Gastric cancer, *SETD2*, Tight junctions, Tumorigenesis.

ABBREVIATIONS: AM: *Atp4b^{Cre}; MYC$^{oe/+}$ mice*; AMS: *Atp4b^{Cre}; MYC$^{oe/+}$; SETD2$^{f/f}$ mice*; EV: empty vehicle; GC: gastric cancer: TJ: tight junctions; TCGA: The Cancer Genome Atlas Programma; RT-qPCR: Real-Time quantitative PCR.

INTRODUCTION

Gastric cancer (GC) is the fourth most deadly cancer in the world, and *MYC* overexpression has been described in over 40% of GC[1,2]. In China, the majority (70%) of GC patients are diagnosed at advanced stages, resulting in poor prognosis[3]. Adenocarcinoma is the most common histological subtype, accounting for 90% of GC cases, while lymphomas and stromal tumors make up the remaining 10%. Adenocarcinoma can be further classified into three subtypes: “intestinal type” (50%), “diffuse type” (33%), and “mixed or unclassified types” (17%)[4-7]. To better stratify GC patients and provide targeted therapies, The Cancer Genome Atlas Programma (TCGA) has categorized GC into four types based on the molecular characteristics: chromosomal instability/intestinal histology (*CIN*, 49.8%), genomically-stable/diffuse histology (*GS*, 19.7%), microsatellite instable (*MSI*, 21.7%), and Epstein-Barr virus-positive tumors (EBV, 8.8%) in 2014[6]. Survival analysis conducted by TCGA revealed that the GC survival times are also dif-

ferent for the various molecular types[6]. Therefore, it is imperative to investigate the pathogenesis and influencing factors contributing to GC development.

Tight junctions (TJ) are intercellular junctions located at the most apical region of cell-cell contacts. They serve two important functions: forming a permeability barrier that restricts the free diffusion of molecules across the intercellular space (gate function) and acting as a membrane fence that prevents the intermixing of apical and basolateral plasma membrane domains (fence function)[8-10]. Abnormalities in TJ, caused by inflammation, mutations, and abnormal signaling pathways, have been associated with the development of the neoplastic phenotype in epithelial cells[11,12]. The main components of TJ are occludins, claudins, and junctional adhesion molecules[13]. The Claudins family plays a key role in various tumors. For example, Claudin-1 promotes colon cancer[14], while Claudin-4 and Claudin-17 affect the invasion of GC cells[15,16]. Increased expression of Claudin-10 has been linked to lung adenocarcinoma development[17]. Studies have demonstrated that the destruction of TJ can promote tumor metastasis[18]. However, the relationship between TJ and GC remains unclear.

SETD2 is a well-known trimethyltransferase that catalyzes the formation of the H3K36me3 modification[19,20]. It plays crucial roles in transcription, DNA repair, and activation of signaling pathways[21-23]. Deficiency of *SETD2* has been linked to various diseases and conditions. In polycystic kidney disease, *SETD2* deficiency leads to Wnt/β-catenin activation and metabolic disturbance, promoting the transformation into renal cell carcinoma[24]. In leukemia, downregulation of *SETD2* enhances the renewal of leukemia stem cells and increases the leukemogenic potential of pre-leukemic cells[25]. Furthermore, in breast cancer, advanced tumors exhibit higher levels of *SETD2* mRNA expression compared to early tumors[26]. Recent research has also found that *SETD2* can facilitate gastric tumorigenesis by damaging gastric epithelial cells, promoting intestinal metaplasia of gastric epithelial cells, and accelerating migration and proliferation of GC cells[27]. Additionally, loss of *SETD2* inhibits the FOXO signaling pathway through phosphorylation facilitation of FOXO1A and FOXO3A proteins, which can lead to gastric tumorigenesis[27]. However, it is still unclear whether *SETD2* has a potential impact on TJ in GC.

Therefore, this research is to further explore the role of *SETD2* in GC and clarify the relationship between *SETD2* and TJ. The study utilized tumor cell lines and a mouse model of stomach-specific *SETD2* depletion together with *MYC* overexpression. The findings show that *SETD2* loss can impair the expression of TJ-related proteins and accelerate the tumorigenesis of GC. This provides a new idea for future precision treatment of GC.

MATERIALS AND METHODS

Mice

All mouse specimens used in this study were obtained from *Atp4b*^{Cre}*; MYC*$^{oe/+}$ mice and *Atp4b*^{Cre}*; MYC*$^{oe/+}$*; SETD2*$^{f/f}$ mice, generously provided by Prof. Li from Shanghai JiaoTong University[27]. *MYC*$^{oe/+}$ mice(24) were mated with *Atp4b*^{Cre} mice to generate *Atp4b*^{Cre}*; MYC*$^{oe/+}$ (AM) mice. *SETD2*$^{f/f}$ mice were mated with AM to generate *Atp4b*^{Cre}*; MYC*$^{oe/+}$*; SETD2*$^{f/f}$ (AMS) mice. All experiments in this study were proved by the Ethics Committee of the Affiliated Tumor Hospital of Xinjiang Medical University (K-2024091).

Cell culture

The human GC cell line SGC-7901 was obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, and 100 µg/ml streptomycin. Culturing was performed in a humidified incubator at 37°C with 5% CO_2. Upon reaching confluence, SGC-7901 cells were trypsinized using 0.25% trypsin at a ratio of 1:3. Subsequently, the subcultured cells were seeded onto 6-, 24-, or 96-well plates at densities of 2×10^5, 5×10^4, and 2×10^3 cells/well, respectively.

RNA isolation and quantitative RT-qPCR

The total RNA was extracted from cultured cells or tissues using the BioTeke RNA extraction kit as instructed. Reverse transcription of the extracted RNA was performed using an RT kit Takara to obtain the cDNA. The obtained cDNA was then used for TB Green real-time PCR analysis. Using *β-actin* for normalization, the data were presented as mean ± SD. Statistical analysis was conducted using Student's *t*-test to calculate the *p*-value.

Western blot analysis and antibodies

The researcher used RIPA buffer that adds protease and phosphatase inhibitors (MCE) to harvest and lyse tissues and cells. Protein concentrations were determined using BCA protein assays. The proteins were separated by 8%-12% SDS-PAGE gels and transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). Millipore was then incubated with primary antibodies, including β-Tubulin (Cell Signaling Technology, 32-2600, Danvers, MA, USA), Claudin-1 (Thermo Fisher Scientific, Cat#3195T, Waltham, MA, USA), followed by secondary antibody treatment. Finally, the blots were developed using an ECL reagent from Thermo Fisher Scientific.

HE staining, IHC staining, and IF staining

Hematoxylin and eosin (H&E) staining, IHC staining, and IF staining were used for tissue sections (5 μm) from AM and AMS. All Standard procedures were followed for all staining. The primary antibodies used in IHC staining included Ki67 (Abcam, ab15580, Cambridge, Cambridgeshire, UK), *SETD2* (LifeSpan, LS-C332416, Newark, Delaware, USA), H3K36me3 (Abcam, ab9050, Cambridge, Cambridgeshire, UK), *MYC* (Abcam, ab32072, Cambridge, Cambridgeshire, UK). The primary antibodies used in IF staining were E-Cadherin (Thermo Fisher Scientific, Cat#3195T, Waltham, MA, USA), ZO-1 (Thermo Fisher Scientific, 402200, Waltham, MA, USA), Claudin-1, anti-Claudin-2 (Abcam, ab53032, Cambridge, Cambridgeshire, UK). Staining intensity was calculated using ImageJ software after completion of HE staining, IHC staining, and IF staining. Microscopy was used to obtain images.

The analysis of RNA sequencing database

Gastric mRNA was obtained from AM and AMS[27]. The RNA-Seq data was downloaded from Gene Expression Omnibus (GEO) (GEO: GSE228037). The researcher applied the DESeq2 algorithm to filter the differentially expressed genes, and the criteria for screening significantly different genes is Fold Change>1.5 or <0.585 and *p*-value<0.05. To gain insights into the biological implications of the differentially expressed genes, gene ontology (GO) analysis was performed using GO annotations downloaded from NCBI (http://www.ncbi.nlm.nih.gov/), UniProt (http://www.unip rot.org/), and the Gene Ontology (http://www.geneontology.org/). Pathway analysis was conducted using the KEGG database (http://www.kegg.jp/) to identify significant pathways of these differentially expressed genes. Fisher's exact test was employed to select the significant pathway based on a threshold of significance defined by a p-value < 0.05.

Statistical analysis

All cell experiments were independently repeated at least three times. Unless otherwise indicated, data are presented as the mean ± SEM. All statistical analyses were performed using GraphPad 9.5 software (225 Franklin Street. Fl. 26 Boston, MA, USA), employing Student's *t*-test (assuming equal variance) and two-way analysis of variance for comparisons between independent groups, statistical significance is considered at $p < 0.05$. Significance levels are denoted as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

RESULTS

Loss of *SETD2* and *MYC* overexpression correlation with poor prognosis in GC patients

To investigate the role of *SETD2* in GC, the GSE54129 database from NCBI was analyzed, and the result showed that *SETD2* mRNA expression levels were lower in GC samples compared to normal samples (Figure 1A). This suggests that *SETD2* serves as a predictor of prognosis in GC. To further explore this, the researcher examined survival information from the GSE15459 database to make a Kaplan-Meier plot. The result showed that high expression of both *SETD2* and *MYC* significantly reduced overall survival (OS) (Figure 1B). Additionally, multiple TCGA databases were investigated to determine the incidence of *SETD2* and *MYC* mutation rates in GC patients. Several TCGA databases showed that approximately 5% of patients had *SETD2* mutations, while 13 had *MYC* mutations (Figure 1C). Interestingly, when analyzing the actual proportion of GC patients with *SETD2* and *MYC* double mutations (2.8%), the researcher observed a higher value than what was expected theoretically (0.65%) (Figure 1D). These results highlighted the correlation between loss of *SETD2*, overexpression of *MYC*, and poor prognosis in GC patients. Further research is warranted to better understand these associations.

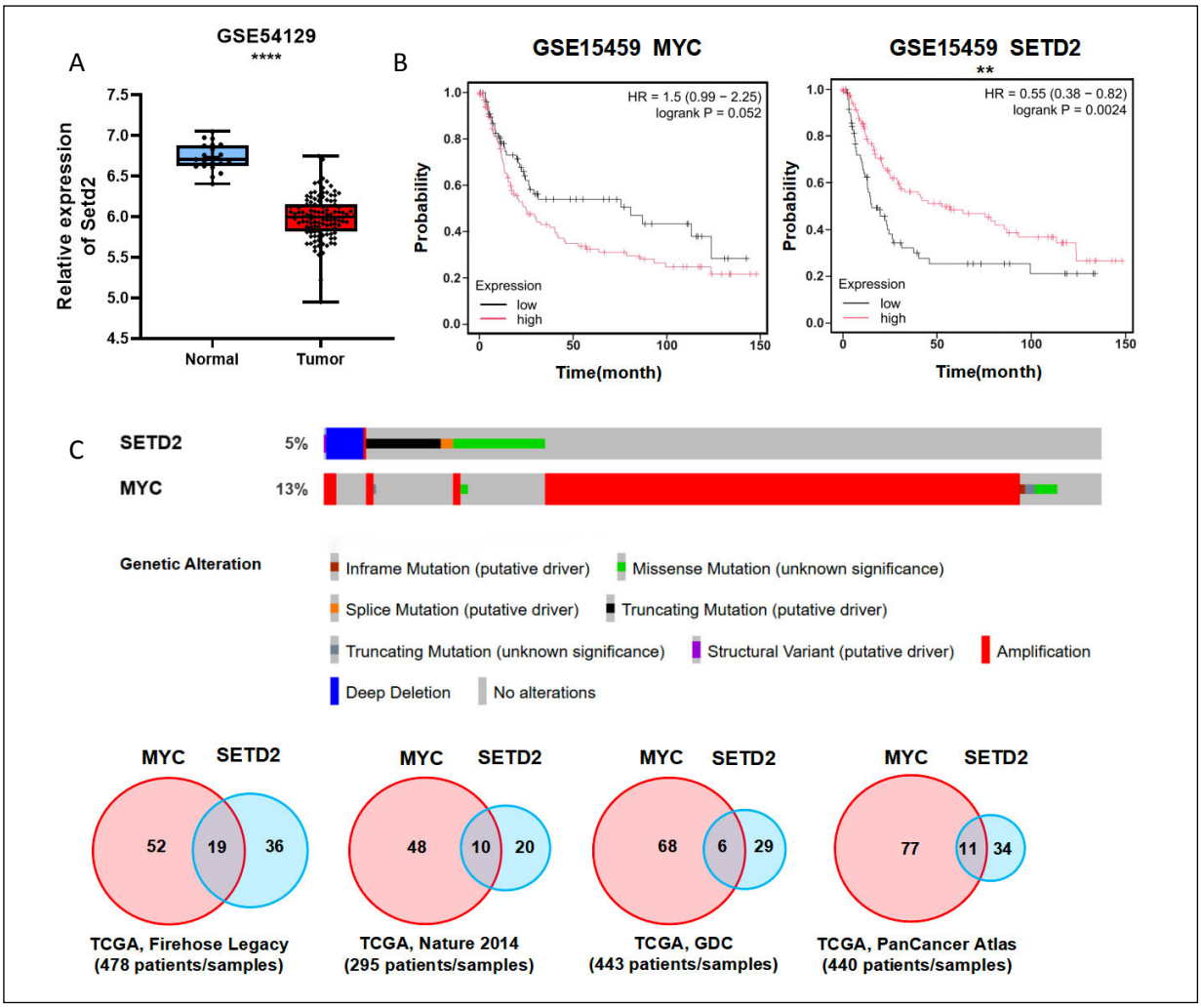

Figure 1. Loss of *SETD2* and *MYC* overexpression correlation with poor prognosis in GC patients (*A*) The level of *SETD2* mRNA expression in normal samples and GC samples (Database: GSE54129). *B*, Kaplan-Meier overall survival curves according to *SETD2* and *MYC* expression were analyzed using GSE15459 databases, respectively. Data were analyzed using Kaplan Meier Plotter (www.kmplot.com). *C*, Distribution of *SETD2* and *MYC* mutations in gastric cancer patients from TCGA databases, respectively. *SETD2* and *MYC* alterations in TCGA databases (Databases: Firehose Legacy, Nature 2014, GDC, and PanCancer Atlas).

Loss of *SETD2* facilitating *MYC*-induced tumorigenesis of GC

To explore the role of *SETD2* in *MYC*-induced GC, this paper utilized specimens from mice with stomach-specific oncogenic *MYC* overexpression and *SETD2* deficiency. $MYC^{oe/+}$ (24) mice were mated with $Atp4b^{Cre}$ mice to generate $Atp4b^{Cre}$; $MYC^{oe/+}$ (AM) mice. $SETD2^{f/f}$ mice were mated with AM mice to generate $Atp4b^{Cre}$; $MYC^{oe/+}$; $SETD2^{f/f}$ (AMS) mice (Figure 2A). Then, the researcher performed Real-Time quantitative PCR (RT-qPCR) to examine *SETD2* mRNA expression of AM and AMS, as we hypothesized that *SETD2* mRNA expression of AMS would be downregulated (Figure 2B). To assess the effects of *SETD2* loss on the stomach, the HE staining was conducted and the result showed that the number of gastric epithelial cells in AMS was larger and disorganized compared to AM (Figure 2C). To further confirm the difference between AMS and AM, IHC and IF staining were performed. The fluorescence area of $E\text{-}Cad^{+}$ in AMS was significantly decreased compared to AM (Figure 2D). IHC staining results showed comparable levels of *MYC* expression between AMS and AM. Moreover, both H3K36me3 and *SETD2* expressions were significantly downregulated in AMS. Importantly, Ki-67 expression was upregulated in AMS (Figure 2E). These results indicate that *SETD2* loss disrupts the normal arrangement of gastric epithelial cells and that both *MYC* overexpression and *SETD2* loss simultaneously accelerate the proliferation of GC cells.

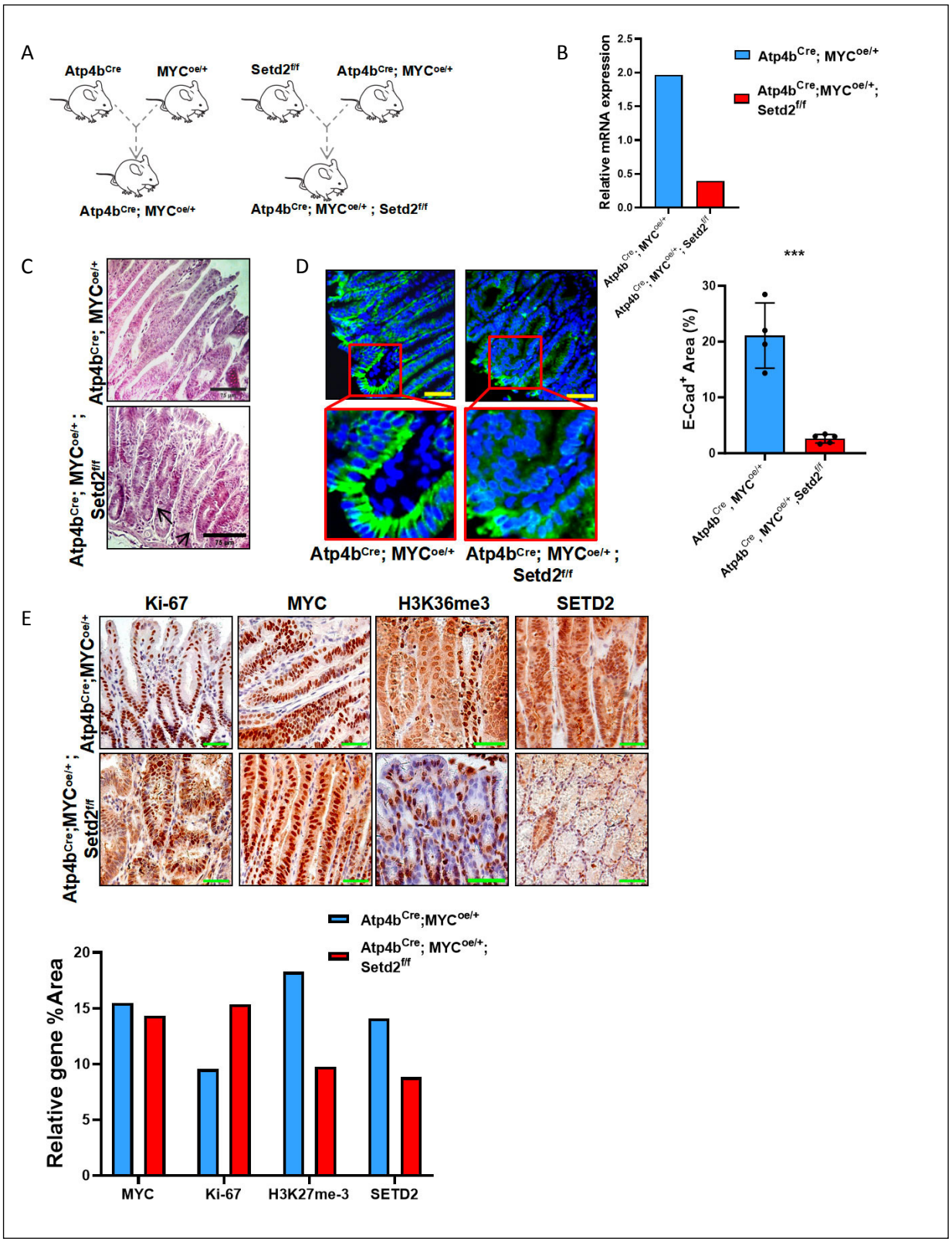

Figure 2. Loss of *SETD2* facilitates *MYC*-induced tumorigenesis of GC (*A*) Construction process of Atp4b^{cre}; MYC$^{oe/(AM)}$ mice and Atp40^{Cre}; MYC$^{oe/+}$; *SETD21** (AMS) mice (*B*) The level of *SETD2* mRNA expression in AM and AMS by using RT-qPCR analysis. Experiments were repeated at least three times. *C*, Gastric tissues were collected from indicated mice (at 12 weeks, the same as below) for HE staining. Black arrows indicate irregularly arranged gastric epithelial cells. Scale bar: 75 um. *D*, IF staining and quantification of E-Cad+ in gastric tissues of AM and AMS. Scale bar: 50 um. *E*, IHC staining and quantification of Ki67, *MYC*, H363Kme3, and *SETD2* in AM and AMS gastric tissues. Scale bar: 50 pm.

The important role of *SETD2* in the TJ

Considering the significant role of TJ in tumor development, such as colon cancer and lung adenocarcinoma[13,16], this paper aimed to investigate the impact of *SETD2* on TJ in GC. The researcher analyzed RNA sequencing (RNA-seq) results (GEO: GSE228037) from gastric tissues of AM and AMS. The result revealed that *SETD2* can influence the upregulation or downregulation of many genes (Figure 3A). KEGG pathway analysis demonstrated a significant difference in TJ-related genes between AM and AMS (Figure 3B). The researcher identified the 111 differently expressed TJ-related genes by applying selection criteria (FC>2 and *p*-value < 0.05), including 15 upregulated genes and 96 downregulated genes (Figure 3C). To validate the influence of *SETD2* loss on TJ pathway, RT-qPCR was performed. The results showed that *Cldn14* and *Amotl2* were significantly downregulated in AMS. Other genes, such as *Tjap1*, *Mapk10*, and *Actr3b,* also exhibited a downregulated trend (Figure 3D). Based on these findings, it can be concluded that *SETD2* plays a crucial role in regulating the expression of TJ-related genes.

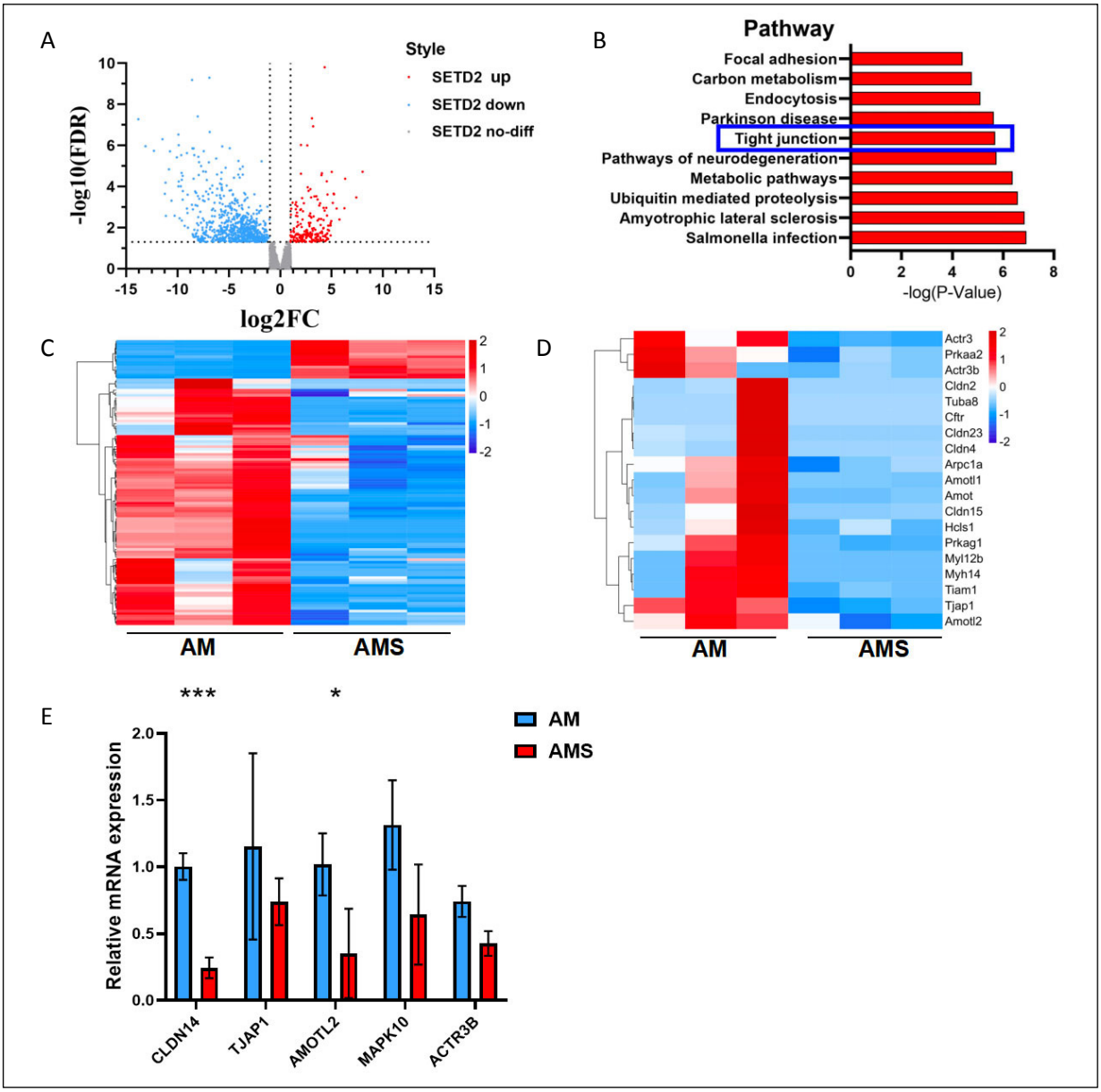

Figure 3. The important role of *SETD2* in the tight junctions (*A*) Volcano map of upregulated or downregulated *SETD2*-related genes. *B*, KEGG pathway analysis of gene expression changes in RNA-Seq data. *C-D*, Heat map of RNA-seq data to compare the TJ-related gene expression of gastric tissues from AM and AMS. *E*, RT-qPCR analysis of mRNA levels of genes associated with the TJ in AM and AMS. The experiment was repeated at least three times, with similar results.

Loss of *SETD2* impairing tight junctions' expression

In previous experiments, the results showed that *SETD2* loss can downregulate the expression of TJ-related genes. To further investigate the effects of *SETD2* deficiency on TJ function in human cells, the researcher used CRISPR/Cas9 system to delete *SETD2* in SGC7901 cells (*SETD2*-KO) and performed RT-qPCR analysis. The result showed that *CLDN24*, *MAPK10*, and *ACTR3B* were significantly downregulated in the *SETD2*-KO group, and the other genes (*CLAND14* and *TJAP1*) also showed a downregulated trend (Figure 4A). Analysis using data from the GEO database (GSE29272) showed that high expression of *ACTR3B* was beneficial to OS in GC patients (Figure 4B). At the same time, IF staining on gastric tissue sections of AM and AMS was performed to test the expression of TJ-related proteins. The results clearly indicated reduced areas stained for Claudin-1, Claudin-2, and ZO-1 in AMS compared to AM samples respectively (Figure 4C-D). In summary, the results provide evidence that the loss of *SETD2* leads to the downregulation of TJ-related genes and subsequently impair the expression of TJ-related proteins.

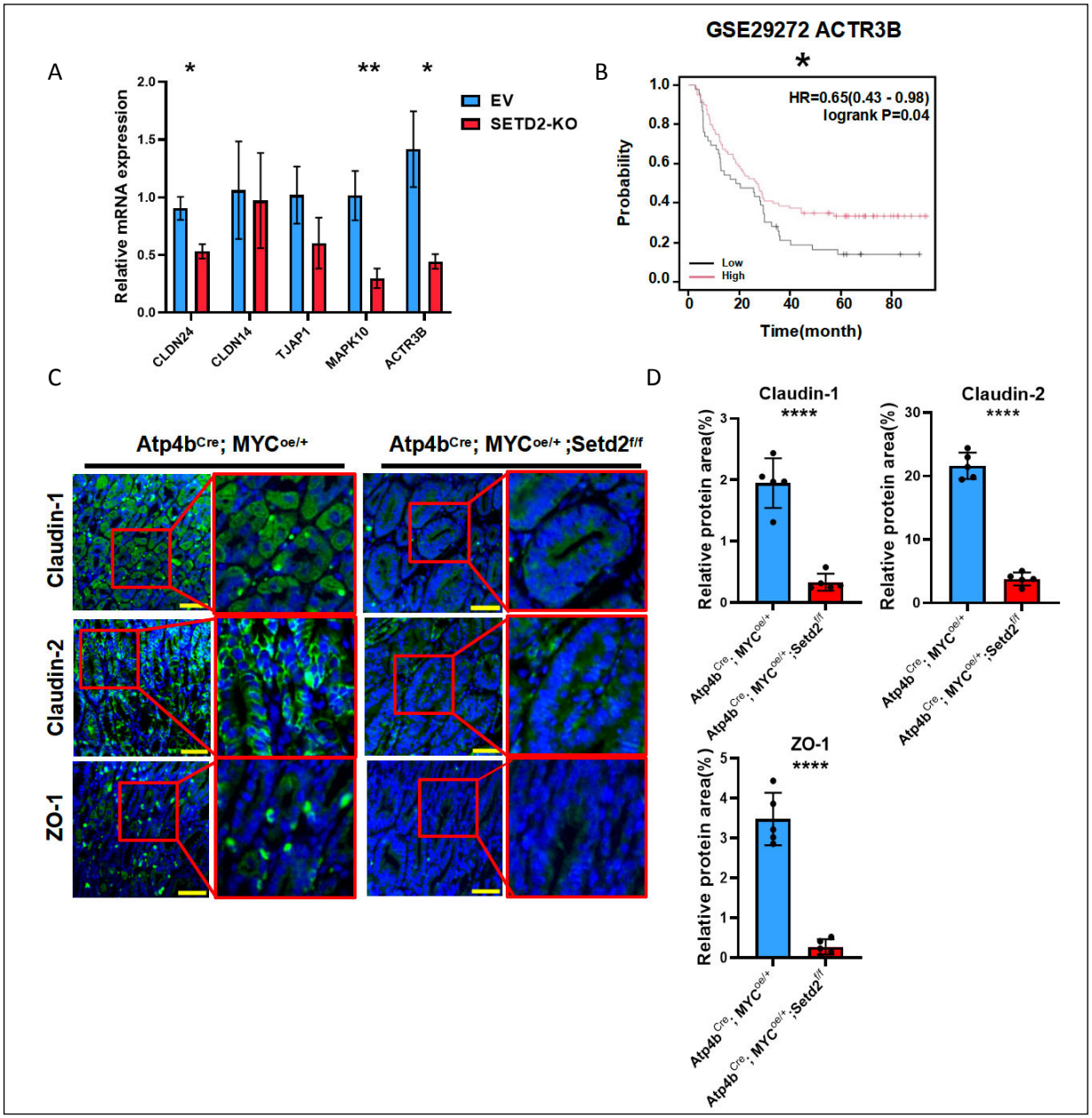

Figure 4. Loss of *SETD2* impairing tight junctions' expression (*A*) RT-qPCR analysis of mRNA levels of genes associated with the TJ in *SETD2*-KO group. Experiments were repeated at least three times, with similar results. *B*, Kaplan-Meier overall survival curves according to ACTR3B expression were analyzed using GSE15459 databases. Data was analyzed using Kaplan-Meier Plotter (http://www.kmplot.com/). *C-D*, IF staining and quantification of Claudin-1, Claudin-2, and ZO-1 in AM and AMS's gastric tissues. Scale bar: 50 um.

The injury of tight junctions promoting the proliferation of human GC cells

To obtain the expression of TJ-related proteins in the cell during *SETD2* loss, western blotting was performed with *SETD2*-KO. The results showed that the expression of TJ-related protein Claudin-1 was significantly down-regulated in the *SETD2*-KO group (Figure 5A). To further evaluate cell viability, a CCK-8 assay was conducted by comparing the *SETD2*-KO group with an empty vehicle (EV) group. The researchers observed that the cell viability of *SETD2*-KO group was significantly higher than that of the EV group (Figure 5B). Meanwhile, another CCK8 assay demonstrated a substantial increase in the number of *SETD2*-KO cells compared to those in the EV group (Figure 5C). These findings demonstrate that *SETD2* loss can impair the expression of TJ-related proteins and substantially increase cell viability and proliferation in human GC cells.

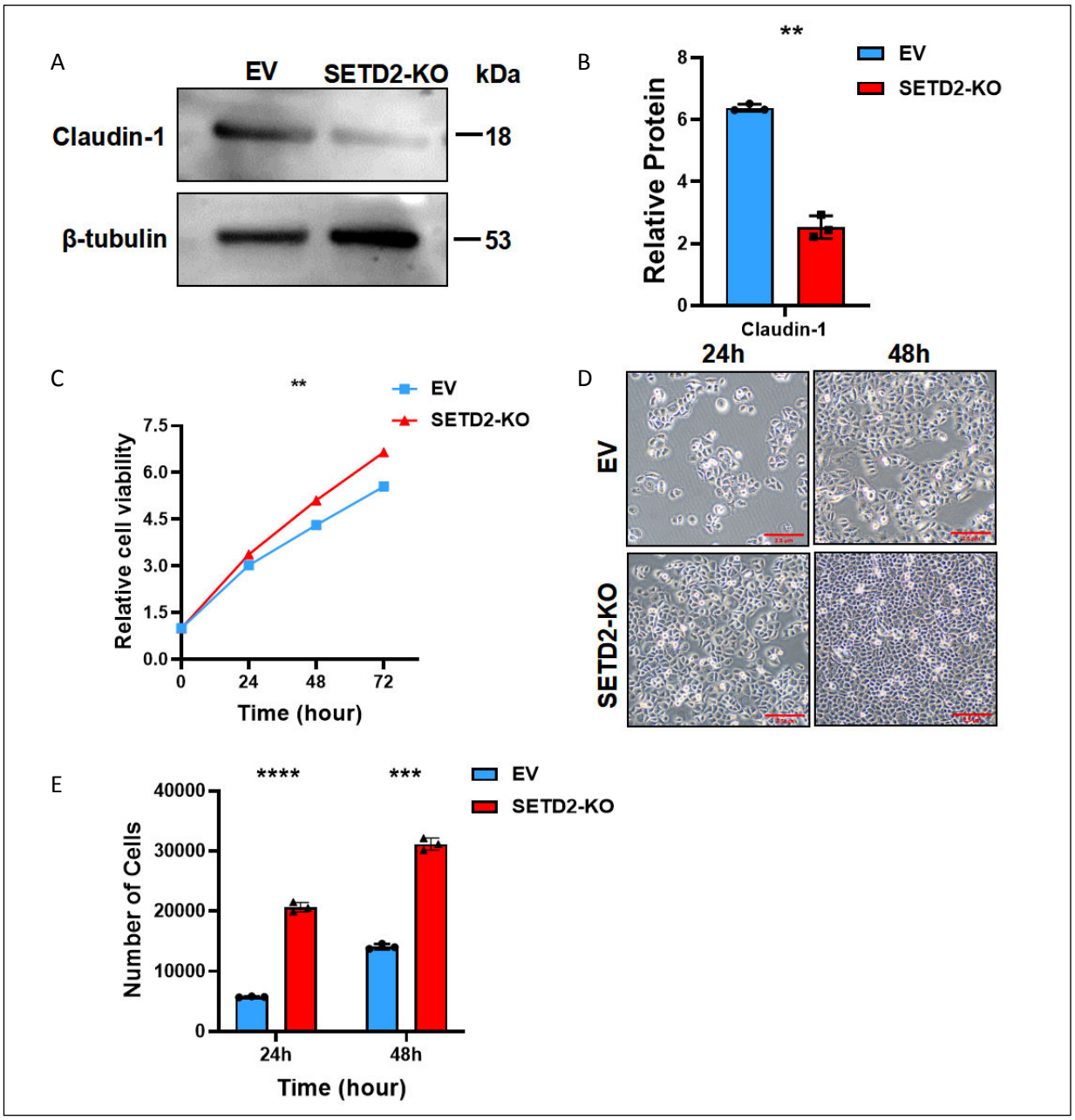

Figure 5. The injury of tight junctions promoting the proliferation of human GC cells (*A-B*) Western blotting analysis of *SETD2*, B-TUBULIN in *SETD2*-KO and EV cells. The experiment was repeated at least three times, with similar results. *C*, CCK8 assay of *SETD2*-KO and EV SGC7901 cells, respectively. The experiment was repeated at least three times, with similar results. *D-E*, Cell counts of *SETD2*-KO cells and EV cells at 24 and 48 hours. Scale bar: 2.5 um.

DISCUSSION

Most GC patients are diagnosed at an advanced stage, and the high incidence of this disease worldwide poses a serious threat to overall patient survival[1,3]. Therefore, it is crucial to understand the mechanism and influencing factors between *SETD2* and TJ to redefine the occurrence and treatment of GC. In this study, the researcher analyzed data from the TCGA and GEO databases and found that loss of *SETD2 and MYC* mutations appeared in a part of the GC patients with worse OS. Some studies have suggested that *SETD2* deficiency promotes gastric tumorigenesis while also being considered to be a predictor of good prognosis in GC[27,28]. Besides, some studies showed that TJ structure was the first barrier to tumor metastasis[29,30], and disruption of TJ-related proteins has been reported to affect the occurrence of GC[15,16]. Therefore, the AM and AMS mouse models were used to explore the relationship between *SETD2* and TJ.

Multiple studies have demonstrated the crucial role of TJ-related genes and proteins, particularly the Claudins family[16,31-35], in the development and metastasis of various tumors through upregulation or downregulation. This research revealed that *SETD2* deficiency can impair the expression of these TJ-related proteins (Claudin-1, Claudin-2, and ZO-1), thereby promoting tumorigenesis and proliferation in GC. These proteins have also been found to facilitate tumor progression in colon cancer and pancreatic cancer[36,37]. We already know that *SETD2* can influence the expression of TJ-related proteins, but the signaling pathway needs to be further explored. For example, *SETD2* deficiency can downregulate the expression of TJ-related proteins (Claudin-1 and E-cad) by inhibiting the SIRT1/FOXO pathway to promote the occurrence of GC2[27,38,39]. The absence of *SETD2* resulted in the down-regulation of DUSP7, which is involved in the inhibition of the RAS/ERK pathway to disrupt the expression of TJ-related protein (ZO-1)[40,41]. Moreover, it has been shown that the damage to TJ can be repaired through Rho flares[42]; however, it is unclear whether overexpression of *SETD2* can also repair TJ. Besides, no reports exist on whether repairing TJ could delay GC progression. These are important questions that should be addressed in future investigations.

In addition, the mechanism of *SETD2* on the expression of TJ-related genes in GC also needs to be found out. This study revealed a tendency for TJ-related genes to be downregulated, particularly *CLDN24*, *MAPK10*, and *ACTR3B,* upon *SETD2* loss. Some studies[43,44] have also shown that TJ-related genes *MAPK10* and *ACTR3B* were involved in tumor regulation through other pathways. For example, in esophageal cancer, the *ZNF471-MAPK10/JNK3* pathway is suppressed by promoter *CpG* methylation[43]. Therefore, it is crucial to determine which specific pathway *SETD2* utilizes to regulate TJ-related genes required to target therapies for tumors caused by *SETD2* deletion. Ma showed that the progression of colorectal cancer caused by *SETD2* and SMAD4 deficiency can be slowed by the ERK inhibitor SCH77298440. It is also worth mentioning that the small molecule drug TIC10 can also stimulate FOXO and inhibit the ERK pathway to inhibit the enhancement of cell proliferation and migration caused by *SETD2* deletion, thus preventing the progression of gastrointestinal tumors[27,45]. Moreover, Liu et al[46] indicated that *SETD2* loss modulates oxidative stress that disrupts the intestinal epithelium and barrier, thereby contributing to inflammatory bowel disease and colorectal cancer, but N-acetyl-L-cysteine suppresses reactive oxygen species to alleviate this process. Thus, the treatment of disease caused by *SETD2* deficiency can be addressed by enhancing or inhibiting signaling pathways downstream of *SETD2*.

CONCLUSIONS

The loss of *SETD2* can disrupt the expression of TJ-related proteins (Cloudin-1, Cloudin-2, and ZO-1) and downregulate the expression of TJ-related genes (*CLDN24*, *MAPK10*, and *ACTR3B*), and promote the tumorigenesis of gastric cancer.

Ethics Approval:

All experiments in this study were proved by the Ethics Committee of the Affiliated Tumor Hospital of Xinjiang Medical University (K-2024091).

Informed consent:

Not applicable.

Availability of Data and Materials:

The data that support the findings of this study are available from the corresponding author upon reasonable request. All mouse specimens used in this study were obtained from Atp4b^{Cre}; MYC$^{oe/+}$ mice and Atp4b^{Cre}; MYC$^{oe/+}$; *SETD2*$^{f/f}$ mice, generously provided by Prof. Li from Shanghai JiaoTong University[27]. All mice were bred and maintained at an animal facility under specific pathogen-free conditions and maintained on a C57BL/6J background, and littermates with the same treatment were used as controls in all experiments.

Conflict of Interests:

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors Contributions:

Haosen Zhao: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptu¬alization. Muyesaier Zakeer: Writing – original draft, Validation, Method¬ology, Investigation, Data curation. Qingzhe Meng: Writing – original draft, Validation, Method¬ology, Investigation, Data curation. Yanli Qu: Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

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