



## ORIGINAL ARTICLE

# Aberrant ASPM expression mediated by transcriptional regulation of FoxM1 promotes the progression of gliomas

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## Abstract

Gliomas are the most common form of malignant tumour in the central nervous system. However, the molecular mechanism of the tumorigenesis and progression of gliomas remains unclear. In this study, we used the GEO database to identify genes differentially expressed in gliomas and predict the prognosis of glioma. We observed that ASPM mRNA was increased obviously in glioma tissue, and higher ASPM mRNA expression predicted worse disease prognosis. ASPM was highly expressed in glioma cell lines U87-MG and U251, and knockdown of ASPM expression in these cells significantly repressed the proliferation, migration and invasion ability and induced G0/G1 phase arrest. In addition, down-regulation of ASPM suppressed the growth of glioma in nude mice. Five potential binding sites for transcription factor FoxM1 were predicted in the ASPM promoter. FoxM1 overexpression significantly increased the expression of ASPM and promoted the proliferation and migration of glioma cells, which was abolished by ASPM ablation. ChIP and dual-luciferase reporter analysis confirmed that FoxM1 bound to the ASPM promoter at -236 to -230 bp and -1354 to -1348 bp and activated the transcription of ASPM directly. Collectively, our results demonstrated for the first time that aberrant ASPM expression mediated by transcriptional regulation of FoxM1 promotes the malignant properties of glioma cells.

## KEYWORDS

ASPM, biomarker, FoxM1, glioma

## 1 | INTRODUCTION

Gliomas are the most common primary malignant tumour of the central nervous system in adults, accounting for 27% of all primary brain tumours and 80% of primary malignant brain tumours.<sup>1,2</sup> The histopathological characteristics and prognosis of gliomas show

significant individual difference. Currently, for first-line treatment of glioblastomas, the NCCN guidelines recommend maximal safe resection with or without radiation therapy and chemotherapy.<sup>3,4</sup> Due to the aggressive and invasive nature of malignant glioma, complete neurosurgical resection is impractical and the residual tumour cells are prone to resistant to radiotherapy and chemotherapy, resulting

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in poor prognosis and high recurrence of glioma patients. Therefore, further clarification of the molecular mechanism of the pathogenesis and progression of gliomas is warranted for the development new therapeutic targets for the disease.

*ASPM* (*abnormal spindle-like microcephaly*) gene, also known as *MCPH5*, is the most common family microcephaly mutation gene involved in the regulation of neurogenesis and cerebral cortical size.<sup>5,6</sup> Intracellular ASPM is mainly distributed in centrosome and spindle microtubule intermediates, and ASPM deficiency leads to spindle assembly and mitotic process disruption.<sup>7,8</sup> In addition, ASPM is also implicated in tumorigenesis and tumour progression.<sup>9-11</sup> In gliomas, the expression of ASPM increased with the glioma grade, and ASPM expression is significantly higher in recurrent glioma than that in primary gliomas.<sup>12</sup> Depletion of ASPM suppresses the proliferation of glioma spheres and promotes cell death.<sup>13</sup> Yet, the underlying molecular mechanisms that mediate the up-regulation of ASPM expression in glioma tissues remain unknown.

FoxM1 is a member of the forkhead box (Fox) transcription factor family.<sup>14</sup> Previous evidence indicated that FoxM1 plays important roles in a variety of biological processes including mitotic G1/S and G2/M phase transition, mitotic spindle integrity, DNA damage repair, angiogenesis and tumour metastasis.<sup>14,15,16</sup> FoxM1 is highly expressed in a variety of solid tumours and contributes to malignant transformation and chemotherapy resistance, suggesting that FoxM1 may serve as a potential antitumour target.<sup>17,18</sup> FoxM1 is also highly expressed in glioblastoma (GBM), and higher FoxM1 expression is associated with worse overall survival in glioma patients. Mechanism studies have indicated that FoxM1 can promote glioma progression by enhancing MMP2 transcription<sup>19</sup> or temozolomide (TMZ) resistance by up-regulating RFC5 expression directly.<sup>20</sup> In an attempt to find potential transcript factors that may play a role in increased ASPM expression in gliomas, we predicted several potential FoxM1 binding sites in ASPM promoter using transcript factor binding site prediction databases. However, whether FoxM1 can directly regulate ASPM expression and whether the FoxM1-ASPM axis contributes to the pathogenesis and progression of gliomas remain largely unclear.

In this study, we aimed to identify genes related to the pathogenesis and progression and prognosis of gliomas based on GEO data sets and investigate the effects of ASPM on the biological functions of glioma cells, as well as the role of FoxM1 in the up-regulation of ASPM expression in gliomas. Our results demonstrated for the first time that FoxM1 can directly activate the expression of ASPM by binding to the promoter, thereby regulating the proliferation, migration and invasion of glioma cells.

## 2 | MATERIALS AND METHODS

### 2.1 | Patient samples

All glioma and normal brain control tissue samples were obtained from patients undergoing neurosurgical resection in Xiangya Hospital of Central South University (Changsha, Hunan, China)

between June 2015 and December 2017. All samples were confirmed by post-operative pathology and classified according to the WHO Classification of the Central Nervous System Tumors. All procedures were approved by the Ethics Committee of Xiangya Hospital of Central South University, and informed consent was obtained from patients.

### 2.2 | Bioinformatic data mining

GSE4271, GSE4290 and GSE45921 were downloaded from the Gene Expression Omnibus database, <http://www.ncbi.nlm.nih.gov/geo/>. 100 glioma samples from GSE4271, 157 gliomas samples and 23 non-tumour samples from GSE4290 and 21 glioma samples from GSE45921 were used to screen genes differentially expressed in non-tumour brain tissue and glioma tissue. Genes with  $|\log \text{fold change}| \geq 1$  and adjusted  $P < .05$  were considered as differentially expressed genes (DEGs).<sup>21</sup> GO functional enrichment analysis and KEGG pathway enrichment analysis were performed on the DEGs using the DAVID online database. The CGGA and TCGA glioma data sets were used for prognostic analysis of DEGs. CGGA expression profile data and clinical information with 225 samples (5 non-tumour brain tissues from epileptic patients and 220 glioma tissues) were downloaded at <http://cgga.org.cn/>. TCGA-LGG and TCGA-GBM mRNA expression data and related clinical information were downloaded at <https://genome-cancer.ucsc.edu/> (version 2015-02-24). 473 LGG patients and 539 patients GBM with complete clinical information were included in the TCGA-LGG data set and TCGA-GBM data set, respectively.

### 2.3 | Cell culture and siRNA transfection

Human glioma cell lines U251-MG and U87-MG were obtained from National Infrastructure of Cell Line Resource of China. Human glioma cell lines Hs683, A172, T98G and U343 were kindly provided by Dr Lv, Jiangxi Cancer Hospital, China. Cells were cultured in DMEM (Gibco, CA, USA) supplemented with 10% (v/v) foetal bovine serum (Gibco, CA, USA) at 37°C in a humidified incubator with 5% CO<sub>2</sub>. U251-MG or U87-MG cells were seeded in 6-well plates at a density of  $2 \times 10^5$  cells/well. After 24-hour incubation, cells were transfected with different siRNAs using RNAiMAX according to the manufacturer's instructions.

### 2.4 | RNA isolation and quantitative real-time PCR

Total RNA was extracted from glioma tissues or cultured cells using RNAiso Plus reagent (Takara, Beijing, China), and then, cDNA synthesis was carried out with PrimeScript™ RT Reagent Kit (Takara, Beijing, China) according to the manufacturer's directions. The mRNA expression level of the target gene was detected using the fluorescence quantitative Real Time PCR Kit (Takara, Beijing, China)

with the Roche LightCycler<sup>®</sup> 480 System. Real-time PCR primers for genes are shown in Table S2.

## 2.5 | Immunohistochemical staining

Paraffin-embedded glioma tissue sections were dewaxed with xylene and then hydrated with gradient ethanol. After antigen repair and blocking, the tissue sections were incubated with ASPM (Santa Cruz, CA, USA)- and FoxM1 (GeneTex, CA, USA)-specific primary antibodies in a wet box at 4°C overnight. Negative control sections were in parallel incubated with non-specific IgG (Sigma, MO, USA). Then, tissue sections were incubated with a secondary antibody for 2 hours at room temperature. After staining with DAB and haematoxylin, tissue sections were observed and photographed under a microscope.

## 2.6 | Western blot analysis

The treated cells were collected and lysed with RIPA lysis buffer (Beyotime, Shanghai, China). The protein concentration was determined using BCA Protein Assay Kit (Beyotime, Shanghai, China). Equal amounts of proteins were separated by SDS-PAGE and transferred to PVDF membranes (Millipore, MA, USA). After blocking, the membranes were immunoblotted with primary antibodies that were specific for ASPM, CyclinD1 and CyclinE (Santa Cruz, CA, USA); FoxM1 (GeneTex, CA, USA); P53, P21 and N-cadherin (Cell Signaling Technology, MA, USA); GAPDH (Proteintech, Wuhan, China); and E-cadherin, Vimentin, MMP1, MMP2, MMP9, RB and CDK2 (Wanlei Biotech, Shenyang, China). After washing with TBST, the membranes were incubated with appropriate secondary antibodies. The membranes were then exposed to enhanced Chemiluminescence-Plus reagents (Beyotime, Shanghai, China). ChemiDoc<sup>™</sup> XRS + chemiluminescence imaging system was used to capture the images. GAPDH protein was used as the internal reference to calculate the relative expression level of the protein by Image Lab 3.0 software.

## 2.7 | Cell proliferation detection

Cell viability and proliferation were measured by CCK8 assay according to the manufacturer's instructions. In brief, cells were seeded in 96-well plates at a density of  $1 \times 10^3$  cells/well. At the indicated time-point, 90  $\mu$ L culture medium and 10  $\mu$ L CCK8 were added into each well. After gently mixing, cells were incubated at 37°C for 1 hour. Subsequently, the absorbance at 450 nm was detected by a microplate reader (Bio-Rad, CA, USA), and the proliferation rate of glioma cells was calculated. Clonal formation assay was used to determine the proliferation ability of glioma cells, and the procedures were carried out according to a previous study.<sup>22</sup>

## 2.8 | Wound healing assay

Wound healing assay was performed according to the previous study.<sup>22</sup> In brief, cells were seeded in 6-well plates at a density of  $5 \times 10^5$  cells/wells and cultured for 24 hours. After transfection for 24 hours, wounds were gently made using a micropipette tip, and the cells were washed with sterile PBS solution to remove floating cells. Then, the cells were cultured with serum-free medium for 24 hours. Cells were photographed at 0 hour and 24 hours after scratch under an inverted microscope.

## 2.9 | Transwell migration assay

The treated glioma cells were collected and resuspended in serum-free medium at a final density of  $5 \times 10^5$  cells/well. 200  $\mu$ L of cell suspension was added to the upper chamber of transwell, and 600  $\mu$ L culture medium containing 15% FBS was added to the lower chamber. After incubation for 24 hours, the medium was removed, and the chamber was washed with PBS. Then, cells were fixed with 600  $\mu$ L methanol for 15 minutes. Methanol was removed, and the cells in the upper chamber were gently wiped with a cotton swab. Subsequently, the cells on the underside of the membrane were stained with 0.1% crystal violet solution for 5 minutes. After washing with PBS, cells were observed and photographed under an inverted microscope.

## 2.10 | Cell cycle analysis

The procedure was according to a previous study,<sup>23,24</sup> and the distribution of cell cycle was determined by flow cytometry (Beckman Coulter, CA, USA).

## 2.11 | Dual-luciferase reporter assay

Human FoxM1-overexpressed plasmid and control plasmid were obtained from GeneChem (Shanghai, China). Human ASPM promoter reporter plasmids and pGL3-Basic vector were purchased from BoChu Biotechnology (Changsha, China). PRL-TK vector was kindly provided by Dr Cui, Central South University, China. Glioma cells were transfected with luciferase reporter plasmid and lysed 24 hours later. Subsequently, the firefly luciferase activity and Renilla luciferase activity were determined with the dual-luciferase assay system (Promega, WI, USA) according to the manufacturer's directions.

## 2.12 | Chromatin immunoprecipitation (ChIP) PCR assay

ChIP experiments were performed with the commercially available Pierce<sup>™</sup> Agarose ChIP Kit (No. 26156) (Thermo Fisher, CA, USA)

according to the manufacturer's instructions. Real-time PCR primers for the ASPM promoter region are shown in Table S3.

### 2.13 | Subcutaneous tumorigenesis experiment of nude mice

Female BALB/c nude mice aged 6 weeks and weighing about 18 g were selected for this experiment. Sixteen mice were randomly divided into sh-con group and sh-ASPM group. ASPM stable knockout U87-MG cells (U87-MG-sh-ASPM cells) and knockdown control U87-MG cells (U87-MG-sh-con cells) were collected and re-suspended in pre-cooled DMEM containing 10% Matrigel and 2% FBS at a final density of  $5 \times 10^7$  cells/mL. Then, mice were injected subcutaneously with 200  $\mu$ L cell suspension. After tumour implantation, animal reactions were observed. Nude mice were weighed every 2 days, and the long and short diameters of tumours were measured. The tumour volume was calculated as previously described.<sup>25</sup> After 21 days of transplantation, the mice were killed and tumour tissues were completely dissected. All of the procedures were conducted according to the guidelines of the Institutional Animal Care and Use Committee of Central South University and were approved by the Institutional Ethics Committee of Central South University.

### 2.14 | Statistical analysis

All of the statistical analyses were carried out using SPSS 19.0 software (SPSS Inc, IL, USA) and GraphPad Prism 5.0 (GraphPad Inc, CA, USA). The data are presented as mean  $\pm$  SD. Student's *t* test was used to analyse the significance of the differences between groups, and one-way ANOVA was used to analyse significance of the differences among groups. Kaplan-Meier survival analysis was used to determine the survival profiles, and log-rank test was carried out to assess the statistical significance of differences.  $P < .05$  was considered statistically significant.

## 3 | RESULTS

### 3.1 | ASPM is related to glioma risk and prognosis

Three gene expression profiles (GSE4290, GSE4271 and GSE45921) were used to identify genes related to the pathogenesis and progression of gliomas. As shown in Figure 1A,B and Table 1, a total of 59 differentially expressed genes (DEGs) were found between normal brain tissues and glioma tissues. GO analysis and KEGG pathway enrichment analysis were performed to explore potential biological functions of DEGs (Table S1). GO analysis showed that DEGs were significantly enriched in cell components such as centrosome, nucleocentric centromere and spindle tubulin and were involved in the biological processes such

as cerebral cortex development, mitosis and DNA repair. KEGG pathway enrichment analysis showed that DEGs were mainly enriched in cell cycle, P53 signalling pathway, progesterone-mediated oocyte maturation and ECM-receptor interaction. Then, we selected twelve genes (*ASPM*, *CCNB2*, *CENPA*, *CENPF*, *COLA2*, *DLGAP5*, *GINS1*, *HSPG2*, *KLHDC8A*, *KIF14*, *LAMC1* and *RRM2*) that were not well studied at present, and verified the expression of these candidate genes by real-time PCR in 30 clinical samples. It was found that *ASPM*, *CCNB2*, *HSPG2*, *KLHDC8A* and *RRM2* mRNA were differentially expressed in glioma tissues of different grades. And among them, *ASPM* changes most significantly (Figure S1). More importantly, further validation in the CGGA and TCGA-LGG data sets and clinical samples also demonstrated that the expression of *ASPM* mRNA was increased with the increase in glioma grades, and the high expression of *ASPM* indicated a worse prognosis (Figure 1C,D). These results suggested that *ASPM* may act as a molecular marker for the diagnosis and prognosis prediction of glioma patients.

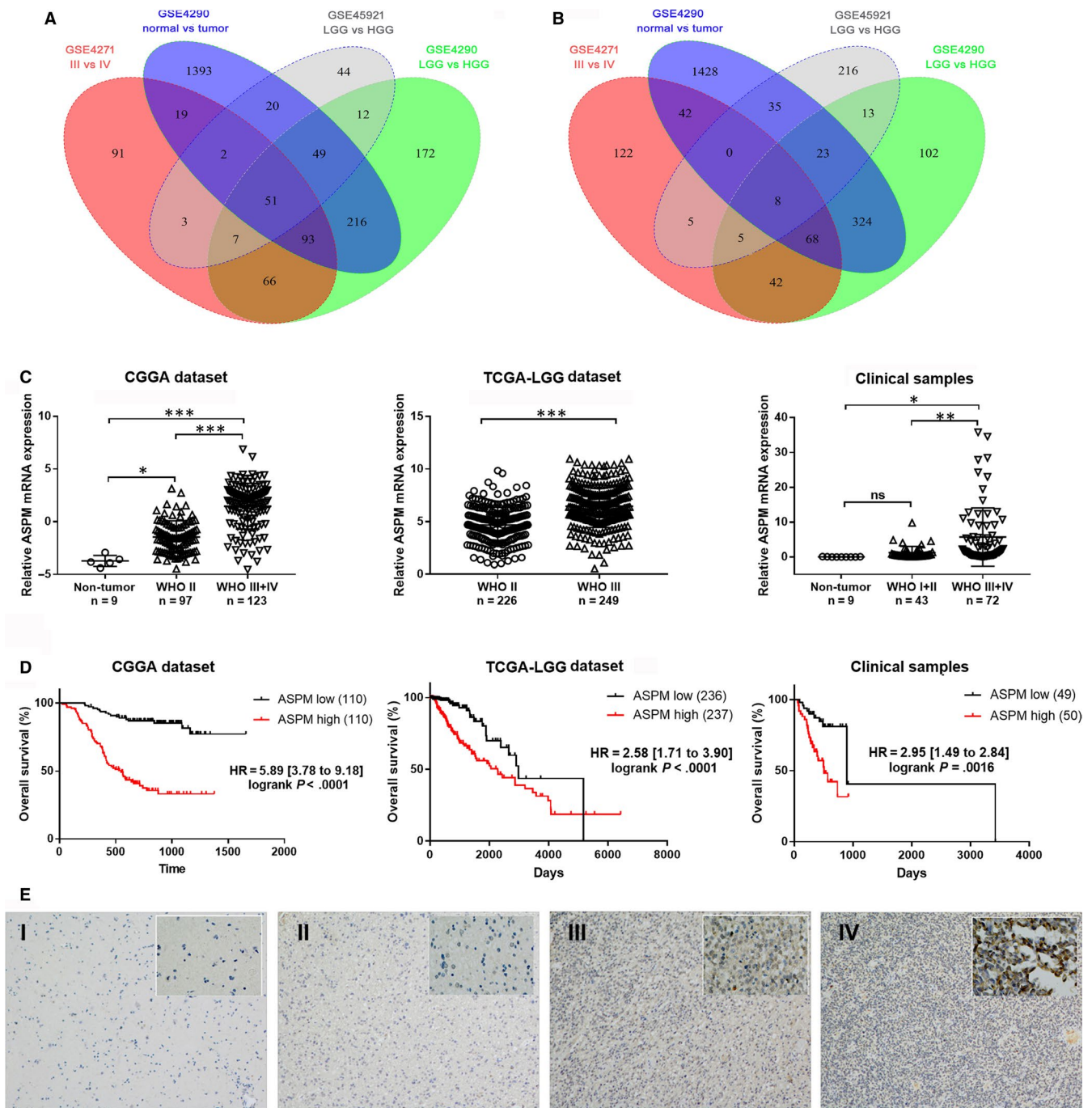
### 3.2 | ASPM inhibition affects proliferation, migration and invasion of glioma cells

In order to investigate the role of *ASPM* in glioma, we primarily detected the expression levels of *ASPM* mRNA in different glioma cell lines. The results showed that *ASPM* was highly expressed in U87-MG and U251 cells and less expressed in Hs683 cells (Figure 2A). As shown in Figure 2C,D, CCK8 and colony formation results indicated that knockdown of *ASPM* by siRNA significantly inhibited the proliferation of U87-MG and U251 cells compared with the si-nc group. Highly aggressive and invasive nature were important malignant phenotypes of glioblastoma. Thus, we performed wound healing and transwell migration assays to evaluate the effect of *ASPM* on the migration ability of glioma cells. Obviously, inhibition of *ASPM* significantly reduced the migration of U87-MG and U251 cells (Figure 2E,F). In addition, compared with the si-nc group, E-cadherin protein remarkably increased in the si-*ASPM* treatment groups, whereas N-cadherin, Vimentin, MMP1, MMP2 and MMP9 protein expression significantly decreased (Figure 2G). Collectively, these results indicated that down-regulation of *ASPM* can inhibit the migration and invasion ability of glioma cells.

### 3.3 | ASPM inhibition induces cell cycle arrest in the mitosis process of glioma cells

Studies have shown that *ASPM* participates in the regulation of symmetrical mitosis and the maintenance of neuronal granulos cells by affecting the cell cycle.<sup>6,26</sup> However, whether *ASPM* is involved in the regulation of the cell cycle of glioma cells remains unclear. To investigate the role of *ASPM* in the cell cycle of glioma, flow cytometry was used to detect the cell cycle of U87-MG and





**FIGURE 1** ASPM is related to glioma risk and prognosis. A, B, Venn diagram showing DEGs in the data set of GSE4290, GSE4271 and GSE45921. C, The expression of ASPM mRNA in normal brain tissues and glioma tissues at different pathological grade in CGGA data set, TCGA-LGG data set and clinical samples; \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . D, Kaplan-Meier plot for overall survival between patients with high and low expression of ASPM mRNA in CGGA data set, TCGA-LGG data set and clinical samples. E, IHC detection of ASPM protein expression in glioma tissues. The sections were photographed at 100× and 400× magnification

U251 cells transfected with si-ASPM or si-nc for 72 hours. As shown in Figure 3A, compared with the si-nc group, ASPM ablation obviously decreased the proportion of cells in S phase and G2/M phase, whereas it increased the proportion of cells in G0/G1 phase. We then detected the effect of ASPM on the expression of cell cycle-related proteins, such as RB, CyclinE, CyclinD1, CDK2, P53 and P21, among which CyclinD1 is an early marker of G0/G1 phase and

CyclinE is a late marker of G0/G1 phase. As shown in Figure 3B, inhibition of ASPM significantly decreased CyclinE protein expression, whereas it increased P21 protein expression, with the protein expression of RB, CyclinD1, CDK2 and P53 having no obvious change. Collectively, these data demonstrated that ASPM may lead to cell cycle arrest of glioma cells by causing abnormal expression of late-G0/G1-related proteins.

## Differentially expressed genes (DEGs)

Up-regulated genes ADAM12, **ASPM**, AURKA, BIRC5, BRIP1, BUB1, BUB1B, C8orf4, CALU, CCNB2, CDC20, CDC45, CDK1, CDKN2C, CENPA, CENPF, CENPN, CENPU, CKS2, COL1A2, DLGAP5, DTL, FANCI, GINS1, GINS2, GTSE1, HMMR, HSPG2, JAG1, KIAA0101, KIF14, KIF20A, KIF23, KLHDC8A, LAMC1, MELK, MYBL2, NCAPG, NDC80, NUSAP1, PARPBP, PBK, RAD51AP1, RRM2, SERPINH1, SHCBP1, SHOX2, SOD2, TACC3, TIMP1, TOP2A

Down-regulated genes ADARB2, ADIRF, CNTN1, MAPK10, NTRK2, PPP1R1A, RTN1, SHANK2

**TABLE 1** Glioma differentially expressed genes screened using GSE4290, GSE4271 and GSE45921 data sets

### 3.4 | FoxM1 expression is positively correlated with ASPM expression and predicts poor prognosis in gliomas

To explore the mechanism of up-regulation of ASPM expression in gliomas, we utilized the JASPAR database and ENCODE database to find the potential transcription factors that may regulate the expression of ASPM, and finally, we obtained 18 transcription factors (EGR1, ETS1, GATA3, NFYA, NFYB, POU2F2, YY1, RUNX3, SP1, SP2, STAT1, BRCA1, CHD1, E2F6, FoxM1, FOXO4, REST and STAT3). To further validate these findings, the correlation between the above transcription factors and ASPM was tested in the CGGA data set, TCGA data set and clinical samples, respectively. We found that *BRCA1*, *CHD1* and *FoxM1* mRNA expression was significantly positively correlated with ASPM mRNA expression in glioma tissues, and the highest correlation coefficient was found between *FoxM1* and ASPM expression (Figure S2, Figure 4A). In addition, the analysis of the CGGA data set, TCGA data set and clinical samples consistently suggested that the expression of FoxM1 was higher in gliomas and predicted a poor prognosis of glioma patients (Figure 4B,C).

### 3.5 | Altering FoxM1 expression affects ASPM expression and proliferation, migration and invasion of glioma cells

Next, we further determined whether FoxM1 has a regulatory effect on ASPM expression. We examined the mRNA and protein expression levels of FoxM1 in different glioma cell lines and found that FoxM1 was highly expressed in U87-MG cells and less expressed in Hs683 cells (Figure 5A). As shown in Figure 5B-D, FoxM1 knockdown suppressed ASPM expression in U87-MG, whereas FoxM1 overexpression increased ASPM expression in U251 cells. FoxM1 overexpression also promoted the proliferation and migration of U251 cells; however, ASPM knockdown reversed the effect induced by FoxM1 overexpression (Figure 5E-G). In addition, FoxM1 overexpression also remarkably decreased E-cadherin expression and increased N-cadherin, Vimentin, MMP1, MMP2 and MMP9 expression, whereas FoxM1 overexpression-mediated changes of EMT-related proteins were overturned by ASPM knockdown (Figure 5H). These results suggested that FoxM1 may

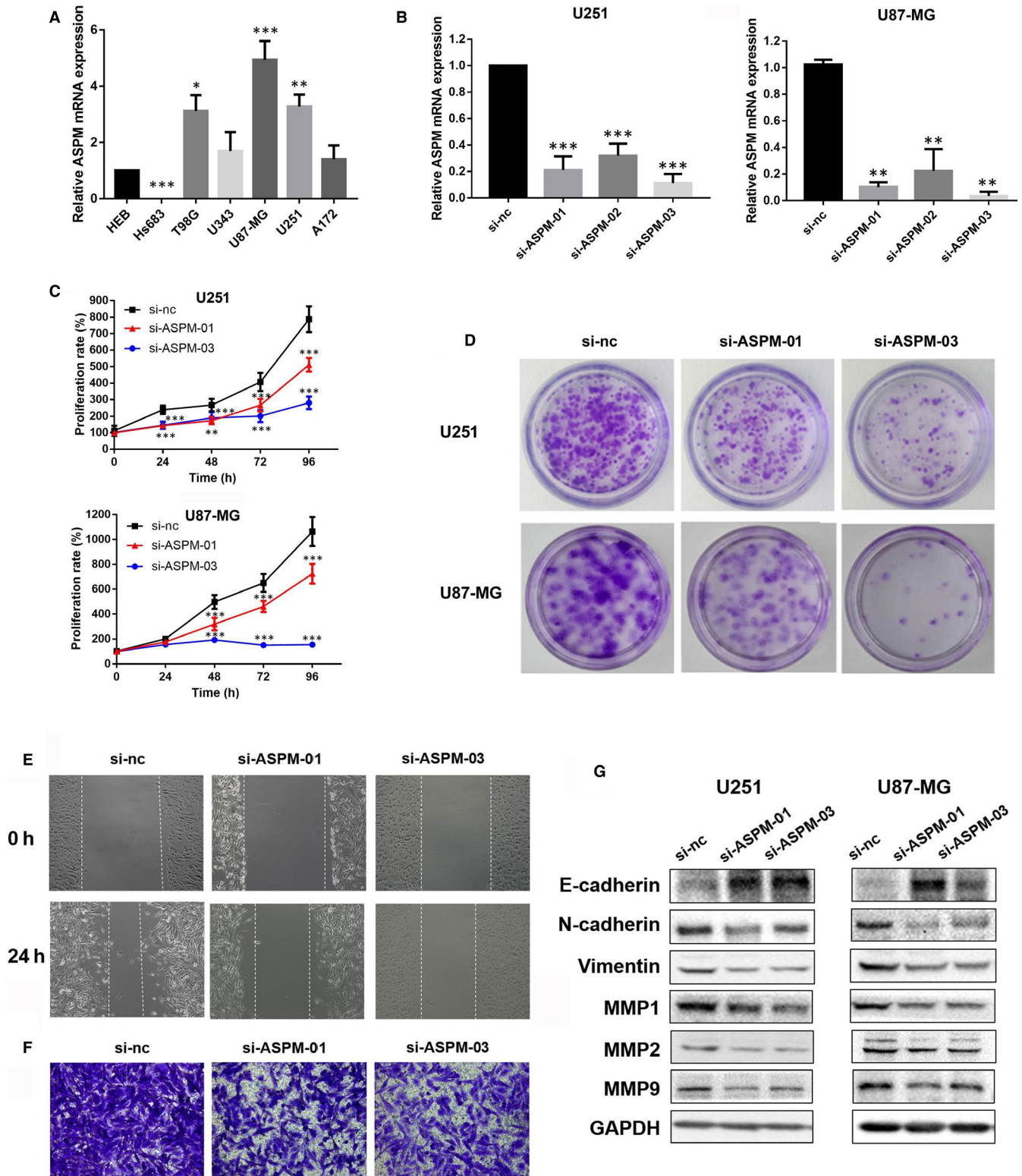
regulate the expression of ASPM, thereby affecting the expression of EMT-related proteins and ultimately regulating the migration and invasion of glioma cells.

### 3.6 | FoxM1 transcriptionally regulates ASPM expression by directly binding to the promoter region

To ascertain whether FoxM1 can directly bind to ASPM promoter region and thus regulate ASPM expression, we first used bioinformatics to predict the potential transcription factor binding sites of ASPM gene promoter and found that ASPM promoter region contains 5 potential FoxM1 binding sites (Figure 6A), and the binding motif of FoxM1 is TGCAAA (Figure 6B). Then, ChIP experiment was used to detect the direct binding of FoxM1 to ASPM promoter. As shown in Figure 6C, FoxM1 was strongly bound to ASPM promoter (-236 to -230 bp, -266 to -260 bp and -1354 to -1348 bp). To observe the effect of FoxM1 on the ASPM promoter activity, firefly luciferase reporter gene vectors containing different lengths of ASPM promoter fragments were constructed as shown in Figure 6D. In Hs683 cells, FoxM1 overexpression significantly activated the luciferase activity of full-length ASPM promoter PA1, whereas its regulatory effect on the truncated ASPM promoter PA4 completely disappeared (Figure 6E). In U87-MG cells, FoxM1 knockdown remarkably suppressed the luciferase activity of full-length ASPM promoter PA1 and truncated ASPM promoters PA2 and PA3, whereas it had no regulatory effect on truncated ASPM promoter PA4 (Figure 6F). These results demonstrated that FoxM1 mainly regulates ASPM promoter activity through binding to ASPM promoter regions (-236 to -230 bp) and (-1354 to -1348 bp).

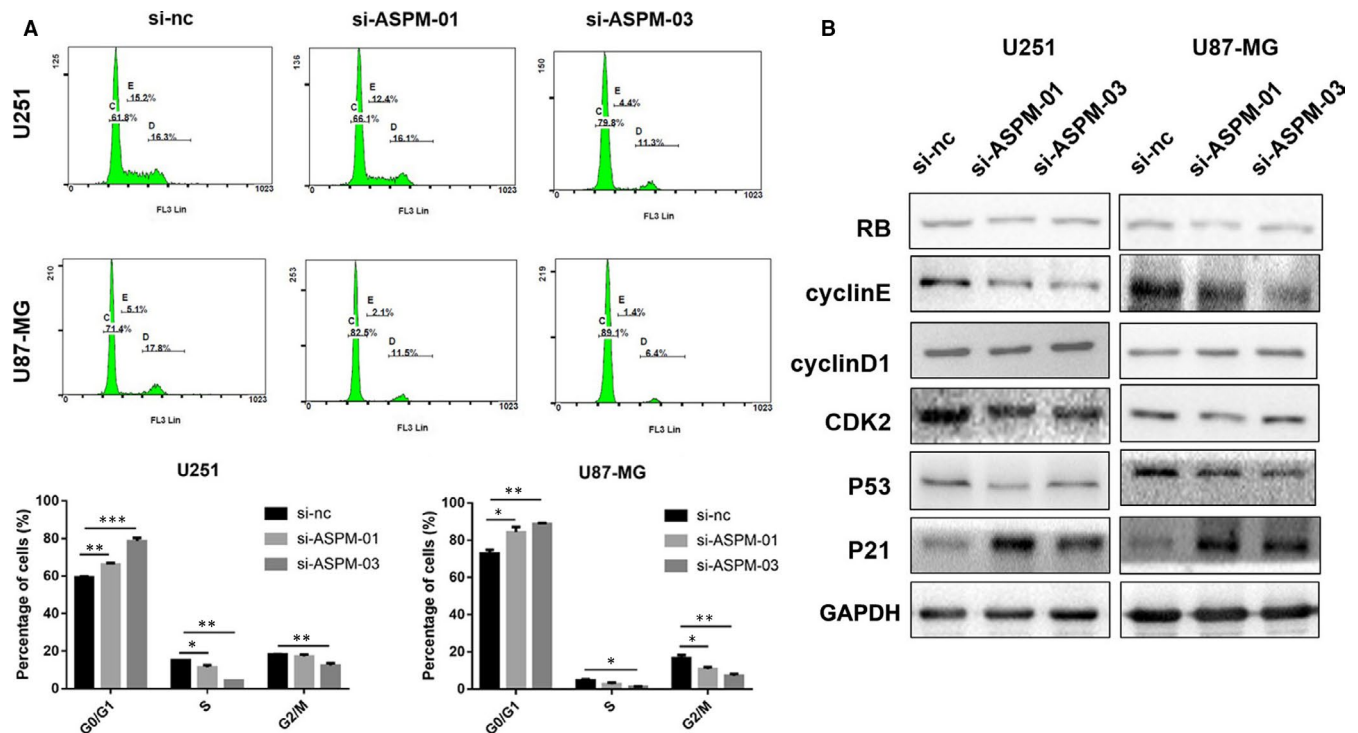
### 3.7 | ASPM is essential for the growth of subcutaneous tumours in nude mice

A xenograft nude mouse model was established to evaluate the effect of ASPM on the growth of gliomas in vivo. Firstly, we used lentiviral infection to establish ASPM stable knockdown U87-MG cells. As shown in Figure 7A, the fluorescence-positive rate of cells was >90% after lentiviral infection. In agreement, compared with U87-MG-sh-nc cells, ASPM expression was significantly lower in U87-sh-ASPM cells (Figure 7B,C). In the subcutaneous tumorigenesis



**FIGURE 2** ASPM inhibition affects proliferation, migration and invasion of glioma cells. A, The expression of ASPM mRNA in different glioma cell lines; \* $P < 0.05$  vs HEB, \*\* $P < 0.01$  vs HEB, \*\*\* $P < 0.001$  vs HEB. The U87-MG and U251 cells were transfected with a control siRNA or ASPM siRNA, (B) the mRNA expression of ASPM was evaluated by real-time PCR, and (C, D) cell proliferation was measured by CCK8 assay and clonal formation assay; \*\* $P < 0.01$  vs si-nc group, \*\*\* $P < 0.001$  vs si-nc group. E, Wound healing and (F) transwell migration tests were used to detect the migration ability of U251 and U87-MG cell. G, The protein expression of E-cadherin, N-cadherin, Vimentin, MMP1, MMP2 and MMP9 in U87-MG and U251 cells was measured by Western blot





**FIGURE 3** ASPM inhibition induces cell cycle arrest in the mitosis process of glioma cells. The U87-MG and U251 cells were transfected with a control siRNA or ASPM siRNA for 72 h. A, B, Cell cycle was detected by flow cytometry, and the protein expression of RB, CyclinD1, CyclinE, CDK2, P53 and P21 was measured by Western blot

model of nude mice, the tumours formed by U87-sh-ASPM cells were significantly smaller than those formed by U87-sh-nc cells, and the proliferation rate of tumours *in vivo* was also remarkably lower (Figure 7D-F). IHC results showed that Ki-67-positive cells in the tumour tissues formed by U87-sh-ASPM cells were significantly less than those formed by U87-sh-nc (Figure 7G). This evidence indicated that inhibition of ASPM can repress the formation and growth of subcutaneous tumours.

## 4 | DISCUSSION

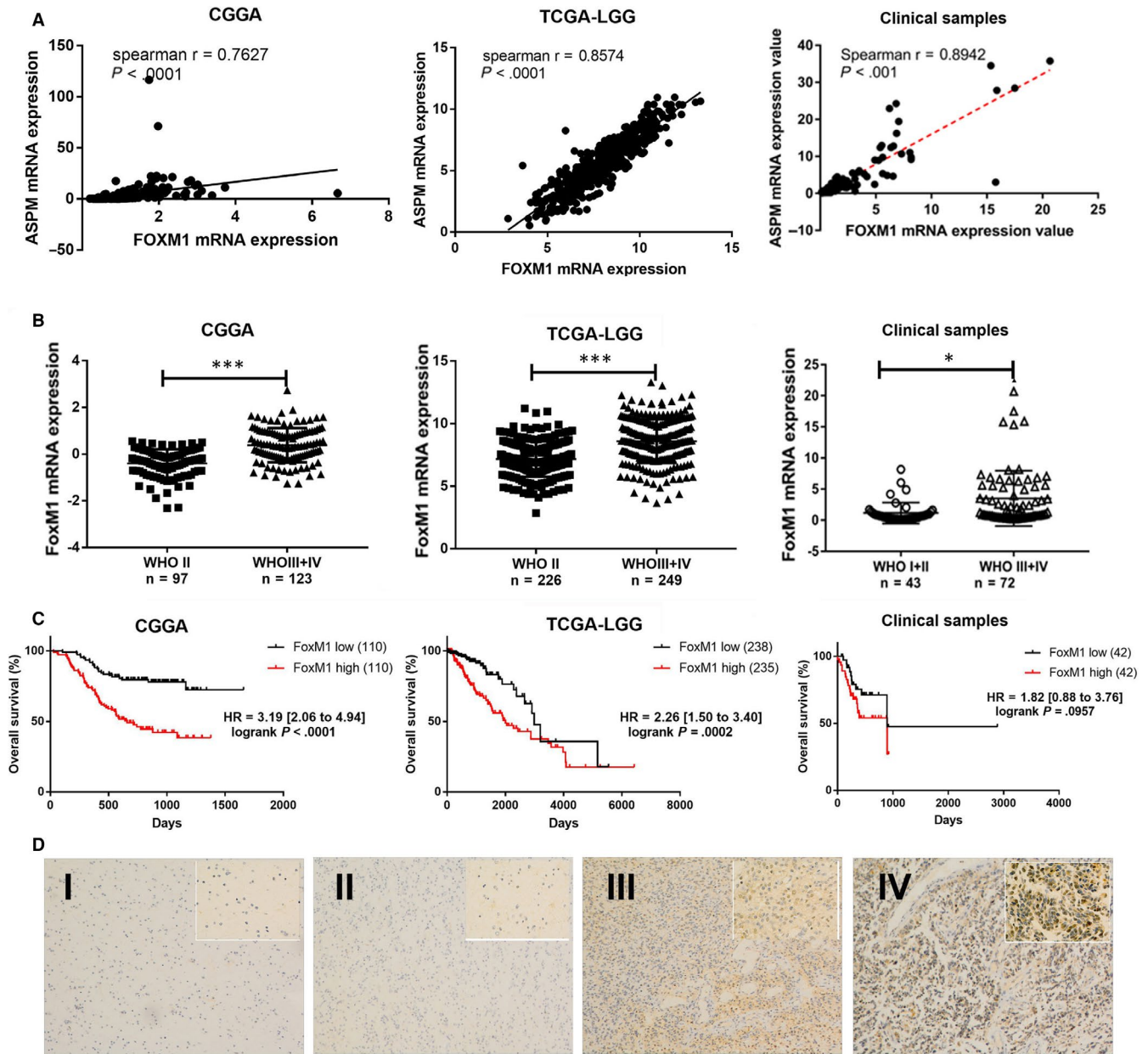
In this study, GSE4290, GSE4271 and GSE45921 were used to screen out 59 differentially expressed genes in normal brain tissues and gliomas of different grades. Through verification of the CGGA data set, TCGA-LGG data set and clinical samples, we found that the expression of ASPM mRNA increased with the increase of glioma grade, and the high expression of ASPM predicted worse prognosis, suggesting that ASPM may function as a molecular marker for the diagnosis and prognosis prediction of glioma patients.

ASPM plays an important role in controlling the neurogenesis and cerebral cortical size, and its homozygous mutation can lead to apoptosis of neural progenitor cells and microcephaly.<sup>5,27</sup> Previous studies have reported that ASPM is mainly distributed in the centrosomes, spindle microtubule intermediates and mid-body<sup>28,29</sup> and plays a vital role in cell division and proliferation in

foetal tissues and human cancer cells.<sup>30,31</sup> In the development of mouse brain, ASPM maintains the symmetrical division and proliferation of embryonic neuroepithelial cells by controlling mitotic spindle orientation.<sup>6</sup> In gliomas, ASPM is elevated in glioma tissues, and ASPM knockdown significantly represses the proliferation of glioma spheres.<sup>12,13</sup> Consistent with previous studies, our results also indicated that ASPM is significantly positively correlated with the pathological grade of glioma and poor prognosis of patients, and knockdown of ASPM could inhibit the proliferation of glioma cells.

Previous studies have found that high expression of ASPM is significantly positively correlated with vascular invasion and early metastasis of hepatocellular carcinoma, and its high expression indicates poor prognosis of hepatocellular carcinoma patients.<sup>32</sup> In metastatic melanoma tissues, ASPM gene is highly expressed, and ASPM overexpression improves the invasion ability of melanoma cells,<sup>33</sup> indicating that ASPM may mediate the invasion and metastasis of tumours. However, the effect of ASPM on the migration and invasion ability of glioma cells has not been reported. In the present study, we found that knockdown of ASPM significantly reduced the migration ability of glioma cells. EMT plays an important role in the invasion and migration of gliomas,<sup>34</sup> and MMPs can promote the migration, invasion and distant metastasis of tumour cells to surrounding tissues by degrading the extracellular matrix.<sup>35</sup> In this study, we found for the first time that knockdown of ASPM can increase the expression levels of E-cadherin protein in glioma cells and decrease the expression levels of N-cadherin



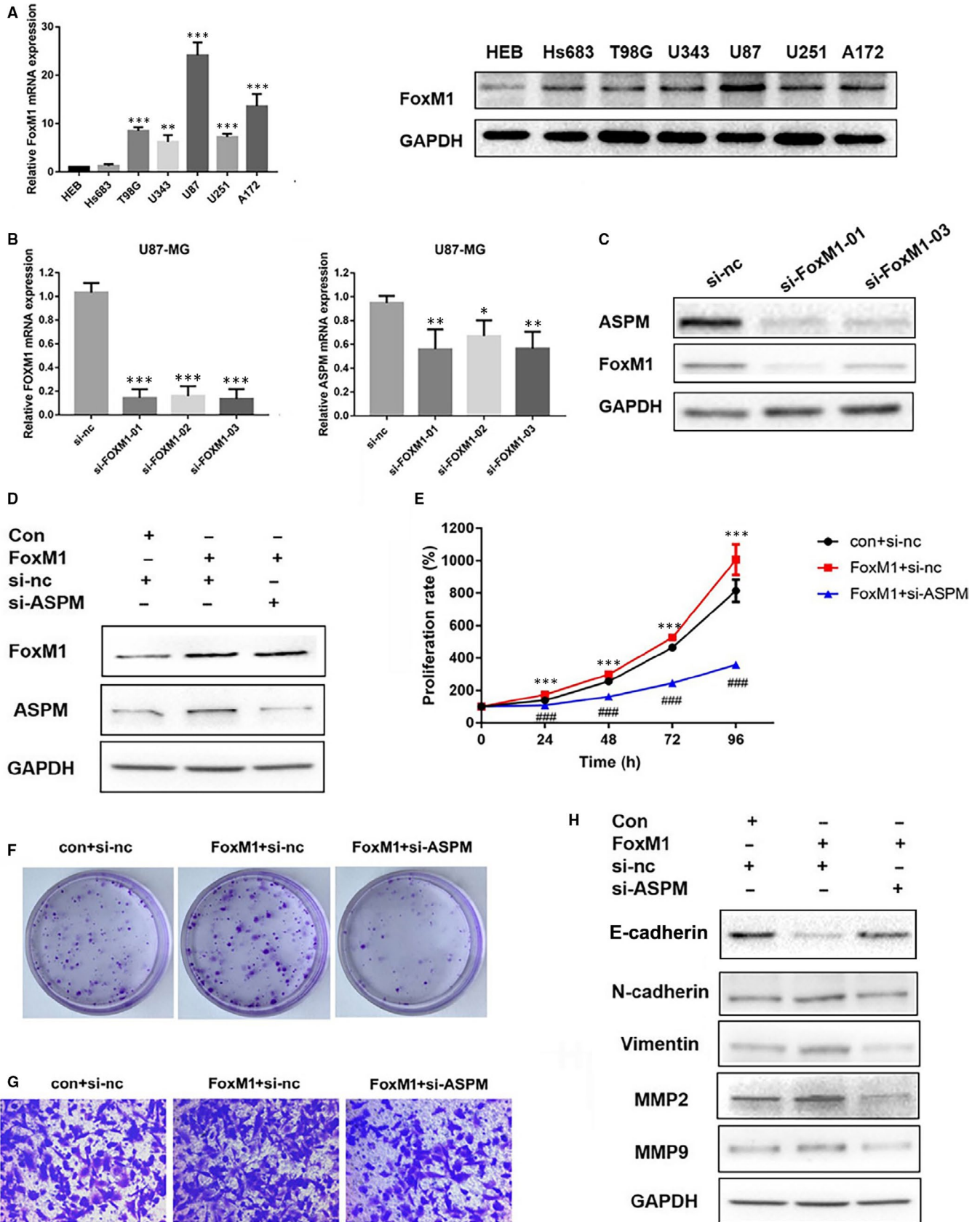


**FIGURE 4** FoxM1 expression is positively correlated with ASPM expression and predicts poor prognosis in gliomas. A, The correlation between *FoxM1* and ASPM mRNA expression levels in CGGA data set, TCGA-LGG data set and clinical samples. B, The expression of *FoxM1* mRNA in normal brain tissues and glioma tissues at different pathological grade in CGGA data set, TCGA-LGG data set and clinical samples; \* $P < 0.05$ , \*\*\* $P < 0.001$ . C, Kaplan-Meier plot for overall survival between patients with high and low expression of *FoxM1* mRNA in CGGA data set, TCGA-LGG data set and clinical samples. D, IHC detection of *FoxM1* protein expression in glioma tissues. The sections were photographed at 100 $\times$  and 400 $\times$  magnification

and Vimentin, MMP1, MMP2 and MMP9 proteins, suggesting that ASPM may affect the migration and invasion of glioma cells by regulating the expression of EMT-related proteins.

Abnormal proliferation of tumour cells is often accompanied by abnormal expression of cell cycle regulators.<sup>36</sup> Previous studies have shown that ASPM is involved in cell cycle regulation.<sup>6,26,29</sup> For example, HCV NS5A protein down-regulates the protein expression of ASPM and induces aberrant mitotic cell cycle through the PKR-p38 signalling pathway.<sup>37</sup> In mouse neuron stem cells, full length of ASPM inhibits CyclinE ubiquitination in the form of a complex with

CDK2/CyclinE, thereby affecting cell cycle duration and participating in stem cell maintenance.<sup>26</sup> In the current work, we found that knockdown of ASPM can induce cell arrest at G0/G1 phase and down-regulate CyclinE expression in glioma cells. CyclinE/CDK2 complex regulates the phosphorylation of RB, thereby functioning as the G1 checkpoint.<sup>38</sup> In addition, it has been reported that ASPM promotes the ubiquitination modification and inhibits the degradation of CyclinE,<sup>26</sup> suggesting that ASPM interference may lead to the cell cycle arrest of glioma cells by regulating the abnormal protein expression at the late G0/G1 phase.

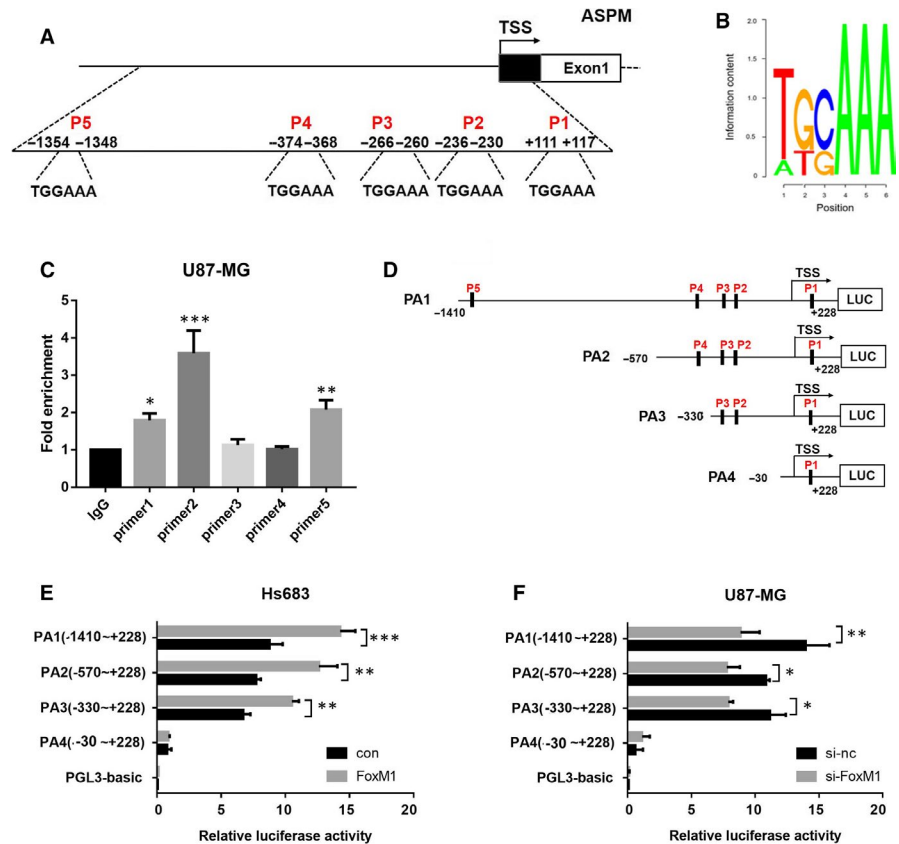


At present, the mechanism that regulates the abnormal expression of ASPM in gliomas remains unrevealed. Studies have found that FoxOs can directly inhibit ASPM expression and thus regulate

the proliferation of neural stem cells.<sup>39</sup> EGFRvIII can up-regulate the expression of ASPM, whereas the overexpression of EGFR inhibitor erlotinib and anti-oncogene PTEN can reverse this effect, indicating

**FIGURE 5** Altering FoxM1 expression affects ASPM expression, proliferation and migration of U251 cells. A, The expression of *FoxM1* mRNA and protein in different glioma cell lines; \* $P < 0.05$  vs HEB, \*\* $P < 0.01$  vs HEB, \*\*\* $P < 0.001$  vs HEB. B, C, The U87-MG cells were transfected with a control siRNA or FoxM1 siRNA, and the mRNA and protein expression of FoxM1 and ASPM were measured by real-time PCR and Western blot; \*\* $P < 0.01$  vs si-nc group, \*\*\* $P < 0.001$  vs si-nc group. The U251 cells were transfected with FoxM1 overexpression vector and a control siRNA or ASPM siRNA. D, Protein expression of FoxM1 and ASPM was measured by Western blot. E, F, CCK8 assay and clonal formation assay was used to measure cell proliferation; \*\*\* $P < 0.001$  vs con group, ### $P < 0.001$  vs FoxM1 + si-nc group. G, Wound healing and transwell migration assays were used to detect the migration ability of U251 cells. H, The protein expression of E-cadherin, N-cadherin, Vimentin, MMP2 and MMP9 in U251 cells was measured by Western blot

**FIGURE 6** FoxM1 transcriptionally regulates ASPM expression by directly binding to the promoter region. A, Bioinformatics predicted the binding region between FoxM1 and ASPM promoter. B, The binding motif of FoxM1. C, ChIP assay was used to detect the binding of FoxM1 to ASPM promoter region in U87-MG cells; \* $P < 0.05$  vs IgG group, \*\* $P < 0.01$  vs IgG group, \*\*\* $P < 0.001$  vs IgG group. D, The firefly luciferase reporter gene vectors with different lengths of ASPM promoter. E, Hs683 cells were transfected with ASPM promoter luciferase reporter gene vectors and FoxM1 overexpression vector for 24 h, and then, the dual-luciferase activity was detected; \*\* $P < 0.01$  vs con group, \*\*\* $P < 0.001$  vs con group. F, U87-MG cells were transfected with ASPM promoter luciferase reporter gene vectors and FoxM1 siRNA for 24 h, and then, the dual-luciferase activity was detected; \* $P < 0.05$  vs si-nc group, \*\* $P < 0.01$  vs si-nc group



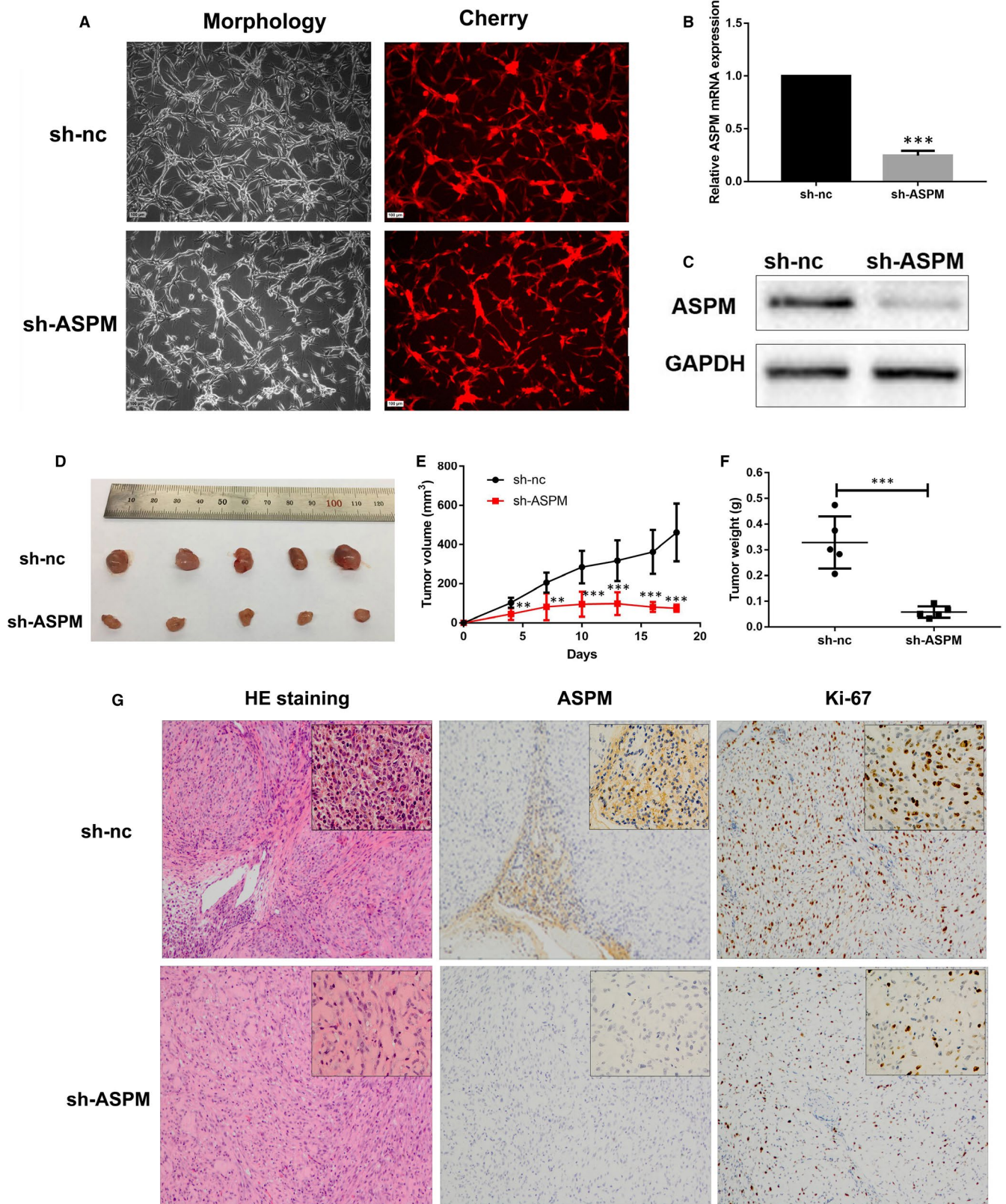
that ASPM expression may be regulated by the PI3K pathway.<sup>13</sup> In order to further investigate the mechanism that induced ASPM up-regulation in gliomas, we utilized the JASPAR and ENCODE database prediction combined with clinical samples and the CGGA and TCGA database validation, and first discovered FoxM1 may be a potential control ASPM expression transcription factors.

FoxM1, a proliferation-specific transcription factor, was first discovered in the cervical cancer cell line HeLa.<sup>14</sup> FoxM1 is highly expressed in embryonic mouse tissues with a high proliferation index, such as thymus, small intestine and testicles, and it is also elevated in a variety of tumour tissues and is associated with tumour malignancy and patient prognosis.<sup>17,18,40,41</sup> It has been reported that FoxM1 is overexpressed in GBM and suggests poor prognosis in glioma patients.<sup>19,42</sup> Similar to previous studies, we also found that FoxM1 is highly expressed in gliomas and its high expression predicts the poor prognosis of glioma patients. In addition, studies have demonstrated that FoxM1 is involved in maintaining tumorigenicity of GBM stem cells, and its mechanism may be related to FoxM1 promoting nuclear localization of beta-catenin.<sup>43</sup>

However, whether FoxM1 plays a role in tumour development by transcriptional regulation of ASPM expression is still unknown. In this study, we demonstrated for the first time that FoxM1 can directly regulate ASPM transcription in glioma cells, thereby affecting their expression and thereby regulating the proliferation, migration and invasion of glioma cells.

In summary, the present study is the first to reveal that ASPM promoted glioma cell proliferation, migration and invasion and tumour growth, which is mediated by the transcriptional activation of FoxM1. In the present study, using the TCGA and CGGA data set analysis, we found that ASPM was highly correlated with FoxM1 expression. Due to the presence of tumour heterogeneity, FoxM1 was unfortunately present only in the DEG list of GSE4290 and GSE45921, and therefore not in the list of 59 DEGs we finally found. This phenomenon suggested that using multiple data sets to screen for DEGs may yield more reliable results but may also lose valuable information. It has been confirmed that FoxM1 is involved in chemotherapy resistance in a variety of tumours.<sup>20,44,45,46</sup> Moreover, studies have shown that FoxM1 can





**FIGURE 7** ASPM is essential for the growth of subcutaneous tumours in nude mice. A, The ASPM stable knockout U87-MG cells (U87-MG-sh-ASPM cells) and the control U87-MG cells (U87-MG-sh-nc cells) were imaged using fluorescence microscopy. B, C, The mRNA and protein expression of ASPM were measured by real-time PCR and Western blot; \*\*\* $P < 0.001$  vs U87-MG-sh-nc cells. D, Subcutaneous tumour was stripped and photographed after transplantation for 18 days. E, Subcutaneous tumour growth curve of nude mice. F, Subcutaneous tumour weight in nude mice. G, The histopathological characteristics and the expression levels of ASPM and Ki-67 protein in subcutaneous tumours of nude mice were detected by HE staining and IHC, respectively



directly promote the expression of RFC5 at the transcription level, thus leading to TMZ resistance independent of MGMT activity.<sup>20</sup> Knockdown of FoxM1 can inhibit the expression of Rad51 and increase the TMZ sensitivity of recurrent GBM cells.<sup>46</sup> These studies suggest that FoxM1 is involved in the regulation of TMZ resistance and drug resistance in glioma. In the future, more experiments are needed to explore the role of the FoxM1-ASPM axis in TMZ chemotherapy resistance.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## AUTHOR CONTRIBUTIONS

W.J. Zeng and Q. Cheng performed the experiments and wrote the paper. Z.P. Wen and J.Y. Wang performed data analysis. Y.H. Chen and J. Zhao collected the clinical samples and performed follow-up. Z.C. Gong and X.P. Chen designed the study. All authors reviewed and approved the final version of the manuscript.

## DATA AVAILABILITY STATEMENT

The data that support the findings described in the current study are available in the article.

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#### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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