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Research

FANCI is involved in the malignant progression of glioma cells by regulating the Akt/Bcl-2 signaling pathway

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Abstract

Introduction Fanconi anemia supplementation group I (FANCI), one of the Fanconi family proteins, may be closely related to the malignant progression of glioma cells. Here, we sought to validate the expression of FANCI in glioma cells and its role in regulating the Akt signaling pathway in the malignant progression of glioma cells.

Methods The expression of FANCI in glioma cells was analyzed by bioinformatics and microarray. Lentivirus transfection was used to regulate the expression of FANCI in glioma cells. CCK-8, EdU, Transwell, and flow cytometry were used to observe the effect of FANCI on the malignant progression of glioma cells.

Results We found that low expression of FANCI inhibited the proliferation, migration and invasion of human glioma cells, and promoted cell apoptosis. However, overexpression of FANCI produced the opposite effect. We also found that low expression of FANCI inhibited the expression of P-Akt and B-cell lymphoma-2 (Bcl-2). Finally, we validated these in vitro results in a xenograft mouse model.

Conclusion FANCI can inhibit the development of glioma by inhibiting Akt/Bcl-2 signaling pathway.

Keywords Glioma · FANCI · Akt/Bcl-2 · Proliferation · Apoptosis

Glioblastoma (GBM) is the most representative aggressive brain tumor in diffuse glioma, with a poor prognosis, and belongs to the WHO grade IV tumor [1]. A survey shows that gliomas with a 5-year survival rate of about 36% [2], and the onset is age-related [3]. After years of medical technology development, therapy methods of glioma from the classic surgical resection combined with radiotherapy and chemotherapy, and gradually advanced in all directions, such as electric field therapy, immunotherapy, targeted therapy, and gene therapy [4, 5]. Unfortunately, the prognosis for gliomas

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remains poor, relapse is inevitable [6]. Therefore, there is an urgent need to explore a molecular marker that could have a role in the development of glioma and drug-resistance therapy.

The goal of chemoradiotherapy is to induce DNA damage beyond the tumor's ability for repair. However, the literature shows that glioma cells have adapted several resistance mechanisms to maintain DNA integrity by combatting DNA damage due to chemoradiotherapy [7]. Contain base excision repair, nucleotide excision repair, base mismatch repair, and single or double-strand breakage repair [8]. Among, the classic DNA interstrand cross-linking repair pathway, also known as the FA (Fanconi anemia) pathway, is characterized by spontaneous DNA breaks and DNA interstrand cross-linking (ICL) phenomena [9]. Therefore, the study of genes related to DNA damage repair is of clinical significance.

Moreover, studies have shown that FA pathway has a research base in susceptibility to cancer [10]. Among more than 30 FA family-related proteins to date, FANCI (Fanconi anemia supplementation group I) has been identified as having a very influential position in ICL repair [11]. FANCI is a nucleosomal localized protein with a molecular weight of 155 kDa, which is involved in the transcription of pre-ribosomal RNA [12]. It can be generated by acting on the DNA sequence on the chromosome, encoding a protein that forms the ID complex together with its molecular partner FANCD2. Through ubiquitination of heterodimers, FANCI recruits effector factors of DNA damage repair (DDR) and plays a crucial role in maintaining genome stability during DNA replication and repair processes [13, 14].

There were more reports that FANCI has to be closely associated with the development of various tumors. In hepatocellular carcinoma, FANCI is closely related to T-helper type 2 cells, which can promote the growth of hepatocellular carcinoma through immune cell escape [15]. A bioinformatics analysis indicated that FANCI may be co-regulated by extracellular matrix receptor and miR-218, leading to a poor prognosis of lung adenocarcinoma by altering the immune microenvironment [16]. Zheng et al. also emphasized that FANCI can cooperate with inosine monophosphate dehydrogenase 2 (IMPDH2) to promote lung cancer cell proliferation through the mitogen-activated protein kinase (MEK)/extracellular regulated protein kinase (ERK)/matrix metalloproteinase (MMP) pathway [10]. It should be noted that FA family is also closely related to brain tumors [17]. However, there are few reports on the expression and regulatory mechanism of FANCI in glioma.

This study aims to analyze the expression of FANCI in glioma tissues and its relationship with WHO grade, prognosis, and recurrence. The effect of FANCI on the biological phenotype of glioma cells was verified by in vitro and in vivo experiments. Also, it verified the function of FANCI knockdown on the tumorigenic ability of glioma in vivo. The results of this study will help to clarify the biological role of FANCI in glioma and its preliminary regulatory mechanism, suggest the regulatory mechanisms of damage repair in glioma cells, and provide a research basis for overcoming chemoresistance in glioma.

1 Materials and methods

1.1 Clinical specimens and Glioma tissue chips

Tissue samples were retrieved from HuanHu Hospital in Tianjin, China, with a total of 5 cases of traumatic brain injury tissue and 42 cases of glioma tissue. Include 9 cases of WHO I grade, 6 cases of WHO II grade, 8 cases of WHO III grade, and 19 cases of WHO IV grade. The patients from whom the samples were derived did not receive any anti-cancer therapies in preoperative treatments, such as chemoradiotherapy. Samples be placed at − 80 °C for later use. All patients signed the informed consent form and were confirmed by the Medical Ethics Committee of Tianjin Huanhu Hospital. Human glioma tissue chip (HBraG160Su01), purchased from Shanghai Xinchao Biotechnology Co., Ltd., contains a total of 152 clinical tissues of different grades of glioma.

1.2 Cell culture

The human glioma cells, namely U251, U87, LN229 and A172, were procured from the China Center for Type Culture Collection (CCTCC) in Wuhan, China. The normal glial cells (NHA) were obtained from the Peking Union Medical College Cell Library. The cells were grown using DMEM medium (DMEM, Gibco) supplementing with 10% fetal bovine serum (FBS, Excell) and 1% penicillin–streptomycin (Gibco) in a 37 °C, 5% CO2 cell culture incubator.

1.3 Cell transfection

Four different FANCI-specific small interfering RNA (siRNA) sequences was used in this study to knockdown the expression of FANCI in glioma cells. Next, pcDNA3.1-FANCI overexpression plasmid targeting human FANCI were synthesized to promote the expression of FANCI. And the effect of transfection verified by real-time PCR and western blot. SiRNA sequences as follow (Shanghai, Genechem):

Sequence 2267: sense (5′-3′): GCACCAGUAUUGGCAUAAATT;
antisense (5′-3′): UUUUUGCCAAUACUGGUGCTT.
Sequence 1292: sense (5′-3′): GCCCAAGUCUUUCUAGAAUTT;
antisense (5′-3′): AUUCUAGAAAGACUUGGGCTT.
Sequence 2097: sense (5′-3′): GGCCUGGUAUAAGAAUACATT;
antisense (5′-3′): UGUAUUCUAUACCAGGCCTT.
Sequence 573: sense (5′-3′): CAGGUGGGAUCAGCAAUAUTT;
antisense (5′-3′): AUAUUGCUGAUCCCACCUGTT.
Negative control sequence: sense (5′-3′): UUCUCCGAACGUGUCACGUTT;
antisense (5′-3′): ACGUGACACGUUCGGAGAATT.

Cells are seeded in 6-well plates one day before transfection to ensure a cell density of 30%-40% at seeding. siRNA or plasmid transfected by Lipofectamine 3000 reagent (Invitrogen, USA), and subsequent cell function experiments after transfection for 48 h. siRNA 1292 was selected as the subsequent lentiviral (Shanghai, Genechem) infection sequence based on the transfection results. After stable transfection 48 h, select the target cells with 0.5 μg/mL puromycin for around 14 days. The plasmid (Shanghai, Genechem) transfection procedure is the same as that of siRNA.

1.4 Quantitative reverse-transcription PCR

Extract total cellular RNA using Trizol reagent. According to the Fastking RT Kit (with gDNase) Usage Guide, synthesize cDNA. Using the SYBR Green Assay Mix Kit on the real-time PCR detection system (Thermo Fisher) perform Real-time PCR experiments. The total working solution system is 20μl, including 0.4μl of forward primer, 0.4μl of reverse primer, 9μl of SYBR reagent, 2μl of cDNA, and 8.6μl of deionized water. The $2^{-\Delta\Delta Ct}$ method determines the relative expression of the target gene. The relative expression of FANCI in each glioma cell was standardized with GAPDH as an internal reference. All experiments were performed in three replicates and were independent. Primer sequences (AG, Accurate Biology) are as follows: FANCI: F: 5′-CTCCTCCAAGGGAAGCAGAAAGA-3′ R: 5′-GGCACAGTGACAACATCCAATAGC-3′.

1.5 CCK-8 cell proliferation assay

About 2,000 cells per well were seeded in a 96-well plate, with five replicate wells a day, placed in a cell culture incubator for 1, 2, 3, 4, and 5 days. Each well-added 100 μl working solution, which mixed 10 μl CCK-8 reagent (Dojindo, Shanghai) and 90 μl DMEM, was next put back incubated for 2 h and then represented proliferative capacity of glioma cells as the absorbance of each well at 450 nm.

1.6 EdU cell proliferation assay

Place suitable coverslips into 24-well plates, then seed around 8,000 cells per well and incubate for 24–48 h. Add into per well with 250 μl the diluted Edu working solution (20 μM) with DMEM, and continue the incubation for two hours. Remove the 24-well plate for 4% paraformaldehyde fixation, and use the EdU cell proliferation kit (Abbkine) to prepare an appropriate amount of staining solution according to the instructions. Observe and photograph under an inverted fluorescence microscope and manipulate the relative proliferation rate of cells using ImageJ software. Value-added rate calculation = number of EdU stained cells/number of DAPI stained cells × 100%.

1.7 Wound healing assay

Inoculate the cells in the 6-well plates at about $1 \times 10^{\wedge 6}$ cells per well to ensure 100% confluency the next day. Scrape the cell monolayer vertically with a 200 µl sterile pipette. The lower serum DMEM (2%FBS) was substituted for complete DMEM (10%FBS). Remove at 0 and 48 h, observe under an inverted microscope, and take pictures. Cell migration area comparison was performed using ImageJ software.

1.8 Transwell migration and invasion assays

For transwell migration assays, aliquots of $2 \times 10^{\wedge 5}$ cells with serum-free DMEM per well in the upper chamber of the Transwell insert chambers (pore size 8 µm, Corning) without Matrigel, add 600µl of DMEM containing 10% FBS in the lower chamber. For invasion experiments, an upper chamber was precoated with Matrigel (Corning). After 48 h, the chamber was removed and fixed with 4% paraformaldehyde 30 min, stained 10 min with crystal violet, and gently wiped the Transwell chamber inner surface with a cotton swab. After properly air-drying, observe it with a microscope and took photography. The number of glioma cells passed through each field of view using ImageJ software processing.

1.9 Flow cytometry

Take about $1 \times 10^{\wedge 6}$ cells after cell transfection 48 h, with 1800 rpm, centrifugation for 5 min. cells were resuspended with 400 µl of 1 × Binding Buffer from apoptosis kit (BD, Biosciences). Then, in the dark conditions add 5 µl of propidium iodide (PI) and 5 µl Annexin V, mix thoroughly, and incubate at room temperature for 10 min. Finally, analyze the apoptosis rate of cells by flow cytometer (Beckman Coulter). Apoptosis rate calculation = (Q1-UR + Q1-LR). Q1-UR represents the proportion of late-stage apoptotic cells in the upper right quadrant, Q1-LR represents the proportion of early-stage apoptotic cells in the lower right quadrant, and Q1-LL represents the proportion of viable cells in the lower left quadrant.

1.10 Western blotting

The extraction of total cell protein using RIPA lysate with protease inhibitors and protein quantification by BCA Protein Quantitation Kit (Beyotime). Prepare a 10% protein gel and polyvinylidene difluoride membranes (0.45 µm PVDF, Millipore, USA). After the protein is transferred to the membrane, the membrane is closed with 5% skimmed milk powder for two hours, and the primary antibody is incubated overnight at 4 °C. The membrane reacted with the corresponding secondary antibody binding reaction at room temperature for 1–2 h the next day, followed by band development using ECL Western Blot luminescence solution (Millipore). In the end, protein band gray value analysis using ImageJ software. The antibodies were as follows: anti-GAPDH (Affinity, 1:1000), anti-FANCI (Abcam, 1:5000), Akt (ABclonal, 1:1000), Phospho-Akt (Ser473) (Always, 1:1000), Bcl-2 (Cell Signaling, 1:1000), Bax (Cell Signaling, 1:1000), HRP-anti-rabbit/mouse (Affinity, 1:5000–1:8000).

1.11 Subcutaneous tumor xenografts in nude mice

BALB/c nude mice (4–5 weeks old) mice raised in the SPF-conditioned animal house. Stably transfected U251 cells with knockdown for FANCI and controls were injected into the right subcutaneous part of each mouse (10 mice in total). After two weeks, observed the growth of the tumor every five days, and sacrificed the mice at four weeks later. Tumors were weighed and treated with 4% paraformaldehyde, and we retained a part of tumor tissue for further histological analysis and RNA extraction. We promise that All experiments involving animals conducted following the protocols of the animal ethics committee approved by the institution.

1.12 Immunohistochemistry

The tissue sections are first deparaffinized and fixed. Follow the instructions for the fluorescent immunohistochemistry kit (Absin). Block with 3%H_2O_2 for 10 min, 5% BSA for one hour at room temperature. Then, incubate with rabbit Ki-67

(Affinity, 1:200), P-Akt (ser 473) (Affinity, 1:100) and Bcl-2 (Affinity, 1:100) primary antibody for overnight at 4 °C in a wet cassette. Hematoxylin dye for 3 min and rinse under running water for 10 min. Soak in gradient ethanol for 5 min each and in dewaxing solution for 5 min. The tissue sectioning was observed and photographed under a microscope.

1.13 Statistical analyses

Data statistical analysis using GraphPad software 8.0. Values from at least three independent experiments. We used the two-tailed unpaired Student's *t*-tests to test the statistical differences between the two groups. The One-way ANOVA test for the between-group comparison. The Kaplan–Meier method and log-rank test plotted the survival curve with statistical differences. A *p*-value less than 0.05 indicates a statistically significant difference.

2 Result

2.1 FANCI is highly expressed in glioma tissues and is associated with poor prognosis

To understand the function of FANCI in glioma development, we first used the GEPIA (http://gepia.cancer-pku.cn/) analysis tool to evaluate the expression level of FANCI in glioma tissues and its correlation with the overall survival of glioma patients. We found that FANCI was highly expressed in glioma tissues (Fig. 1A) and was associated with poor prognosis in gliomas (Fig. 1B). When FANCI had higher expression in primary and recurrent gliomas, the survival time of patients was significantly shorter (Fig. 1C, D). The FANCI expression had positively correlated with WHO grade (Fig. 1E). Therefore, high levels of FANCI expression are associated with progressive WHO grade in glioma patients.

To validate the results of the above public bioinformatics database analysis, we performed immunohistochemical staining (IHC) analysis using human glioma tissue microarray, chi-square analysis of the differences between clinicopathological features and FANCI expression, and qRT-PCR analysis of FANCI expression level in clinical glioma tissues. Tissue microarray results showed that FANCI expression increased with increasing glioma grade (Fig. 1F), and high FANCI expression had inversely correlated with overall survival and disease-free survival (Fig. 1G, H). FANCI was also associated with recurrence status and different grades of glioma (Fig. 1I, J). The chi-square analysis results of the differences between the clinicopathological characteristics and the expression of FANCI in 152 of glioma microarray are shown in Table 1. The results showed that the age ≥ 40 ($\chi^2 = 6.848$, $P < 0.009$), grade III-IV ($\chi^2 = 16.882$, $P < 0.001$), high level of PD-L1 expression ($\chi^2 = 4.746$, $P < 0.029$) were statistically significant with FANCI expression ($P < 0.05$). Furthermore, Cox regression analysis showed that age ≥ 40, grade III-IV, and Ki67 high expression were independent factors predicting the prognosis of glioma patients (Table 2, $P < 0.05$). In glioma clinical samples, FANCI mRNA levels were higher in high-grade than low-grade glioma tissues (Fig. 2A). Generally speaking, these results initially hint that FANCI is involved in the genesis and progression of glioma cells.

2.2 Knockdown FANCI expression on glioma cells

To further verify the effect of FANCI on glioma cell development, we first verified the expression of FANCI in glioma cell lines (NHA, LN229, U251, U87, A172). The results showed that FANCI was highly expressed in LN229 and U251 cells and relatively poorly expressed in A172 and U87 cells (Fig. 2B–D). Therefore, cells with high expression of FANCI (LN229/U251) were selected for knockdown experiments. Cells with low FANCI expression (A172) were used in overexpression experiments. Next, we designed a siRNA sequence targeting FANCI, and transfection experiments showed that the si-1292 sequence had a better knockdown effect on glioma cells. Therefore, we selected the si-1292 sequence as a lentiviral template and successfully constructed glioma cells with stable low expression of FANCI. It was labeled KD-FANCI (Fig. 2E–G). Cellular function assays were performed in un-knockdown versus knockdown FANCI cells to evaluate the role of FANCI in the malignant progression of glioma cells.

2.3 Knockdown of FANCI expression inhibits the proliferation, migration and invasion of glioma cells, and promotes apoptosis

To evaluate the effect of FANCI on glioma cell survival and proliferation, we performed EdU and CCK-8 assays. According to the results, the knockdown of FANCI resulted in a statistically significant change in OD450 values and the rate of

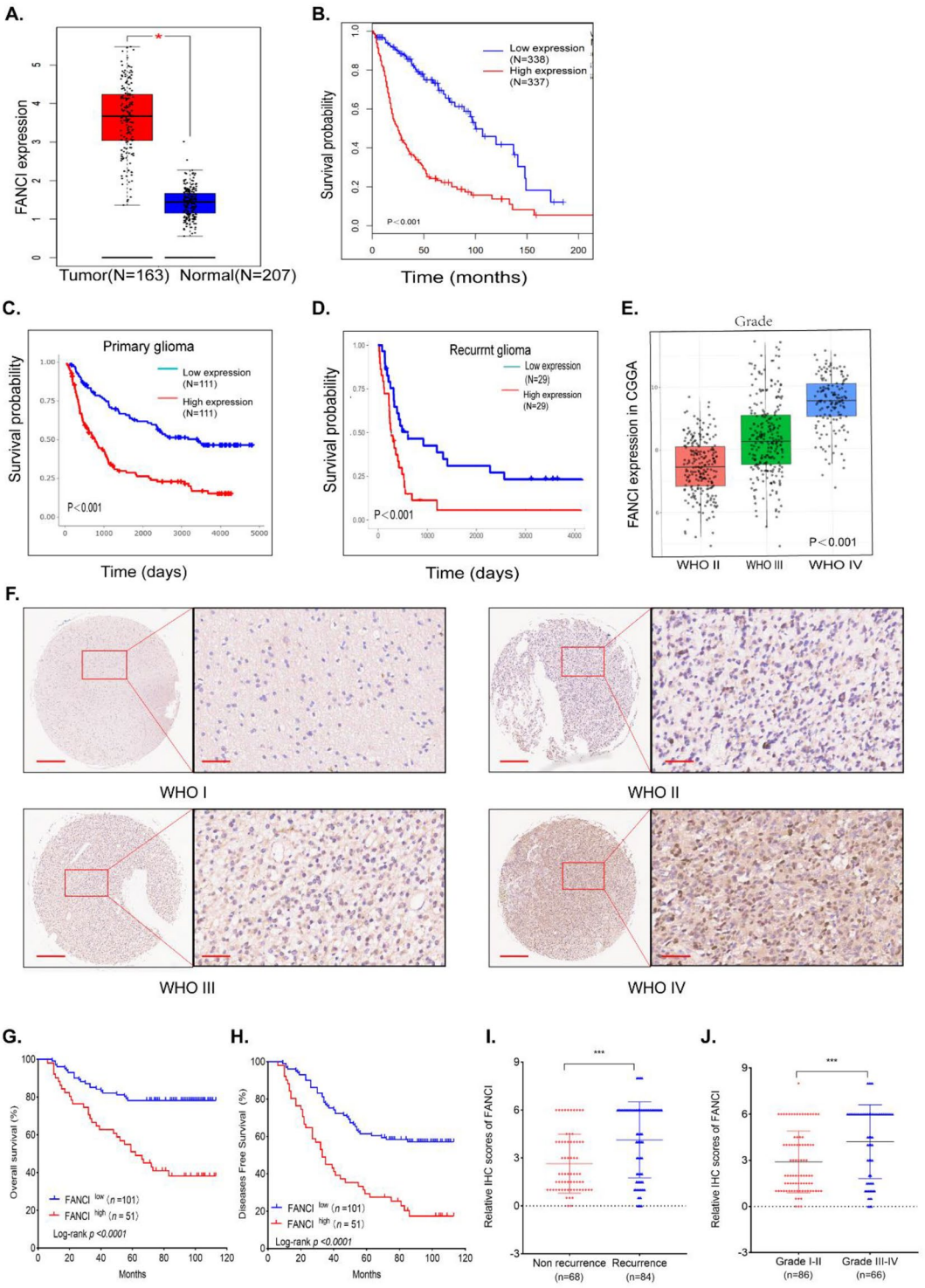

Fig. 1 FANCI is highly expressed in gliomas and is associated with poor prognosis in patients. **A** Expression of FANCI in GBM in the TCGA database. **B** The relationship between FANCI expression and clinical prognosis of glioma in TCGA database. **C**, **D** High of FANCI expression in both primary and recurrent gliomas is associated with poor prognosis. **E** The CGGA database showed that FANCI was positively correlated with the WHO grade of glioma. **F** The results of immunohistochemistry assay showed that FANCI expression was positively correlated with WHO grade of glioma (scale bar = 200 μm). **G–J** The GBM microarray indicates that high levels of FANCI expression indicate a poor prognosis, and the expression is differential in recurrent tumors and WHO grade. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

Table 1 Correlation between FANCI expression and clinicopathological characteristics (N = 152)

	variables	FANCI expression		total	χ^2	*p* value
		low	high			
Age (year)					6.848	0.009**
	< 40	48	13	61		
	≥ 40	53	38	91		
Sex					0.161	0.688
	Female	37	17	54		
	Male	64	34	98		
Grade					16.882	< 0.001***
	I-II	69	17	86		
	III-IV	32	34	66		
ki67					0.160	0.689
	Low	51	24	75		
	High	50	27	77		
EGFR					0.337	0.561
	Low	57	26	83		
	High	43	24	67		
PDL-1					4.746	0.029*
	Low	75	29	104		
	High	26	22	48		

* $p < 0.05$; ** $p < 0.01$
*** P < 0.001

Table 2 Prognostic Value of FANCI Expression with Overall survival in Patients with Gliomas

Variables	Univariate analysis			Multivariate analysis		
	HR	95%CI	*p* value	HR	95%CI	*p* value
Sex (female/male)	1.684	0.930–3.049	0.085	–	–	
Age (< 40/ ≥ 40)	3.971	1.942–8.119	< 0.001*	3.618	1.727–7.578	0.001*
Grade (I-II/ III-IV)	14.267	7.136–28.523	< 0.001*	15.030	7.186–31.432	< 0.001*
Ki67 (low/high)	0.500	0.288–0.866	0.013	0.392	0.216–0.712	0.002*
EGFR (low/high)	1.106	0.647–1.892	0.713	–	–	–
PDL1 (low/high)	1.078	0.608–1.909	0.798	–	–	–
FANCI (low/high)	3.499	3.023–6.053	< 0.001*	–	–	–

*Statistically significant (p < 0.05)

Edu-positive cells in both LN229 and U251 cells (Fig. 2H–I). To evaluate the effect of FANCI on the migration ability of glioma cells, we performed a scratch healing assay and a transwell assay. Wound-healing assay results showed that FANCI knockdown significantly reduced cell migration distance (Fig. 3A). Furthermore, the results of the transwell assay showed that the number of cells passing through the chamber membrane in the KD-FANCI group was significantly lower than that in the control group (Fig. 3B). Similarly, the invasion assay, which evaluated the effect of FANCI on the invasiveness of glioma cells, also showed a significant reduction in the number of cells crossing the Matrigel-containing compartment membrane in the KD-FANCI group compared with the NC group (Fig. 3C). In addition, flow cytometry assesses the effect of FANCI on glioma cell apoptosis. The results show that FANCI knockdown increased the apoptosis rate of LN229 and U251 cells (Fig. 3D). These results indicated that inhibition of FANCI expression reduced proliferation, migration, and invasion, and induced apoptosis of glioma cells.

To further investigate the effect of FANCI overexpression on the proliferation, migration, invasion, and apoptosis of glioma cells, we overexpressed FANCI in A172 cells using plasmid transfection technique and labeled it as OE-FANCI, and verified the increased expression of FANCI in A172 cells (Fig. 4A, B). With the same Edu, CCK8, cell scratch, cell invasion, Transwell, and apoptosis assays. The results showed that the overexpression of FANCI promoted the proliferation (Fig. 4C,

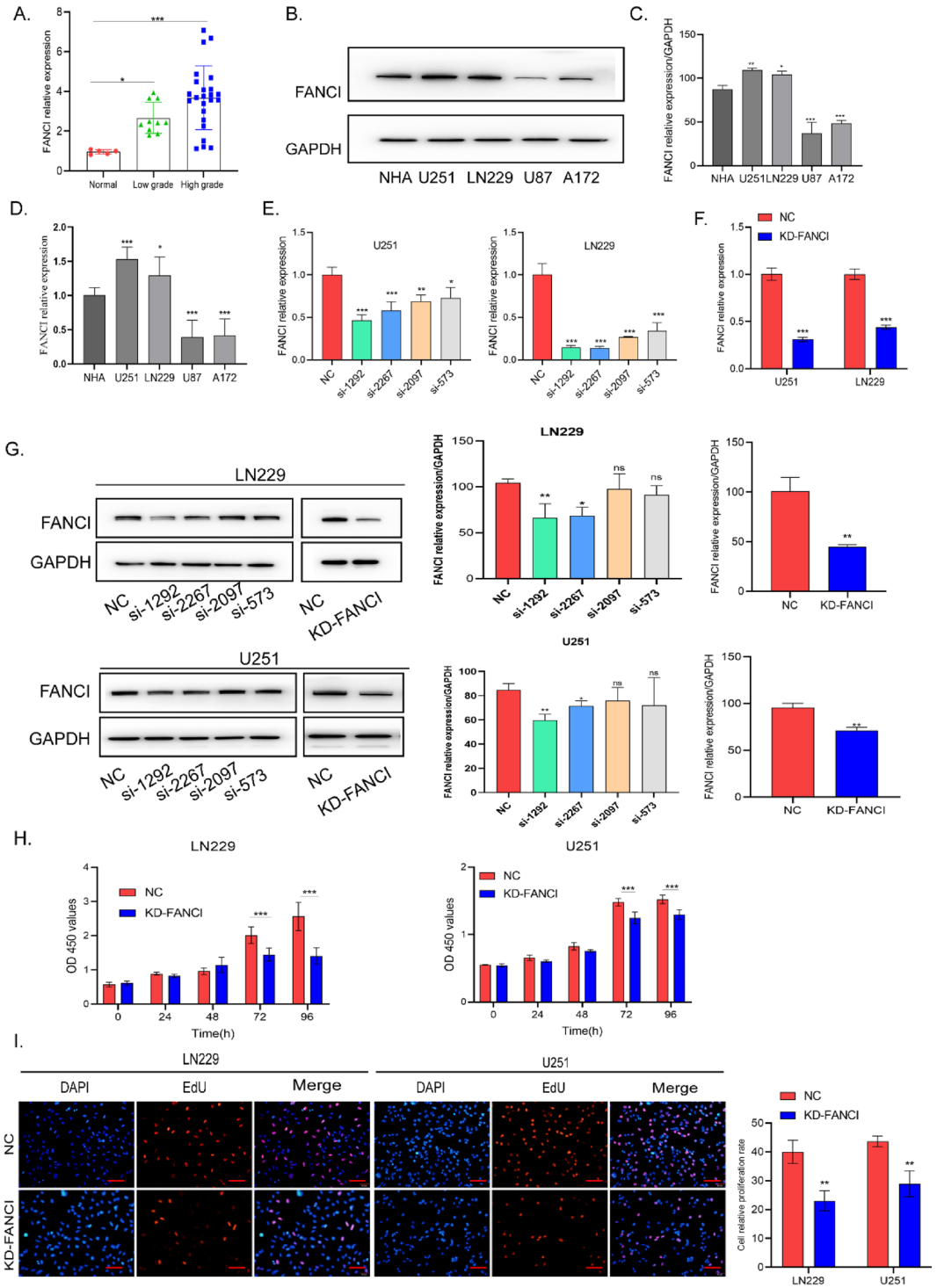

Fig. 2 Expression of FANCI in glioma specimens and cells. The low level of FANCI expression inhibits the proliferation of glioma. **A** FANCI is more expressed in low/high grade gliomas than in non-tumor tissues. **B-D** Representative FANCI protein and mRNA expression in different glioma cell lines. **E** siRNA interference in U251/LN229 cells. **F** lentiviral transfection in U251/LN229 cells. **G** Western blotting was used to verify siRNA interference and lentiviral transfection (KD-FANCI). **H, I** CCK-8 assay and EdU proliferation assay were used to analyze the effect of FANCI knockdown on the proliferation ability of glioma cells, respectively (scale bar = 100 μm). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

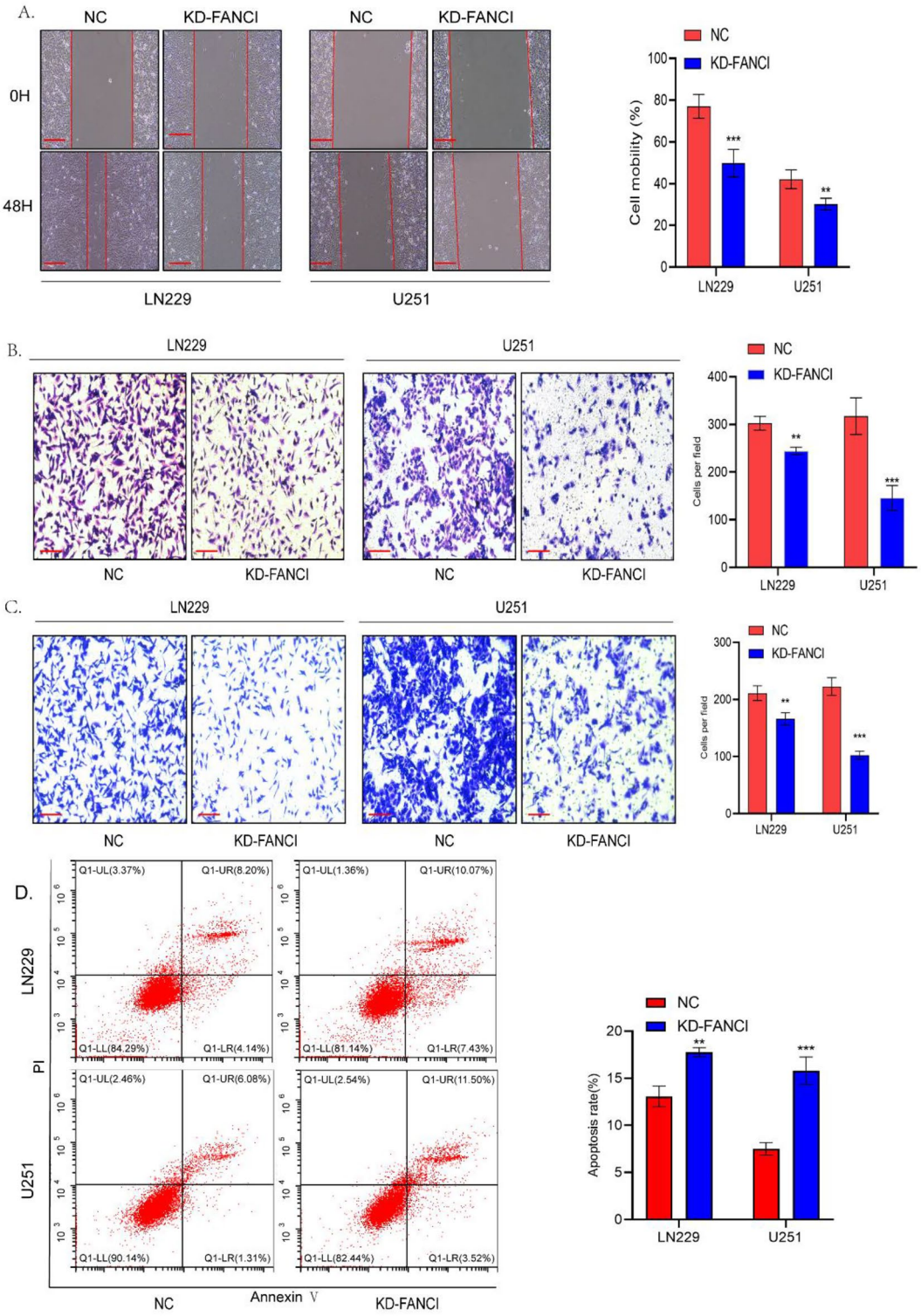

Fig. 3 Low levels of FANCI expression inhibit the migration and invasion of glioma cells and promotes apoptosis. **A**, **B** The scratch assay and the Transwell assay were used to detect the migration ability of LN229 cells and U251 cells after low expression of FANCI (scale bar = 100 μm). **C** Transwell invasion assay was used to analyze the motility of LN229 cells and U251 cells after low expression of FANCI (scale bar = 100 μm). **D** Apoptosis assay analysis of the effect of FANCI on the apoptosis of LN229 cells and U251 cells. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

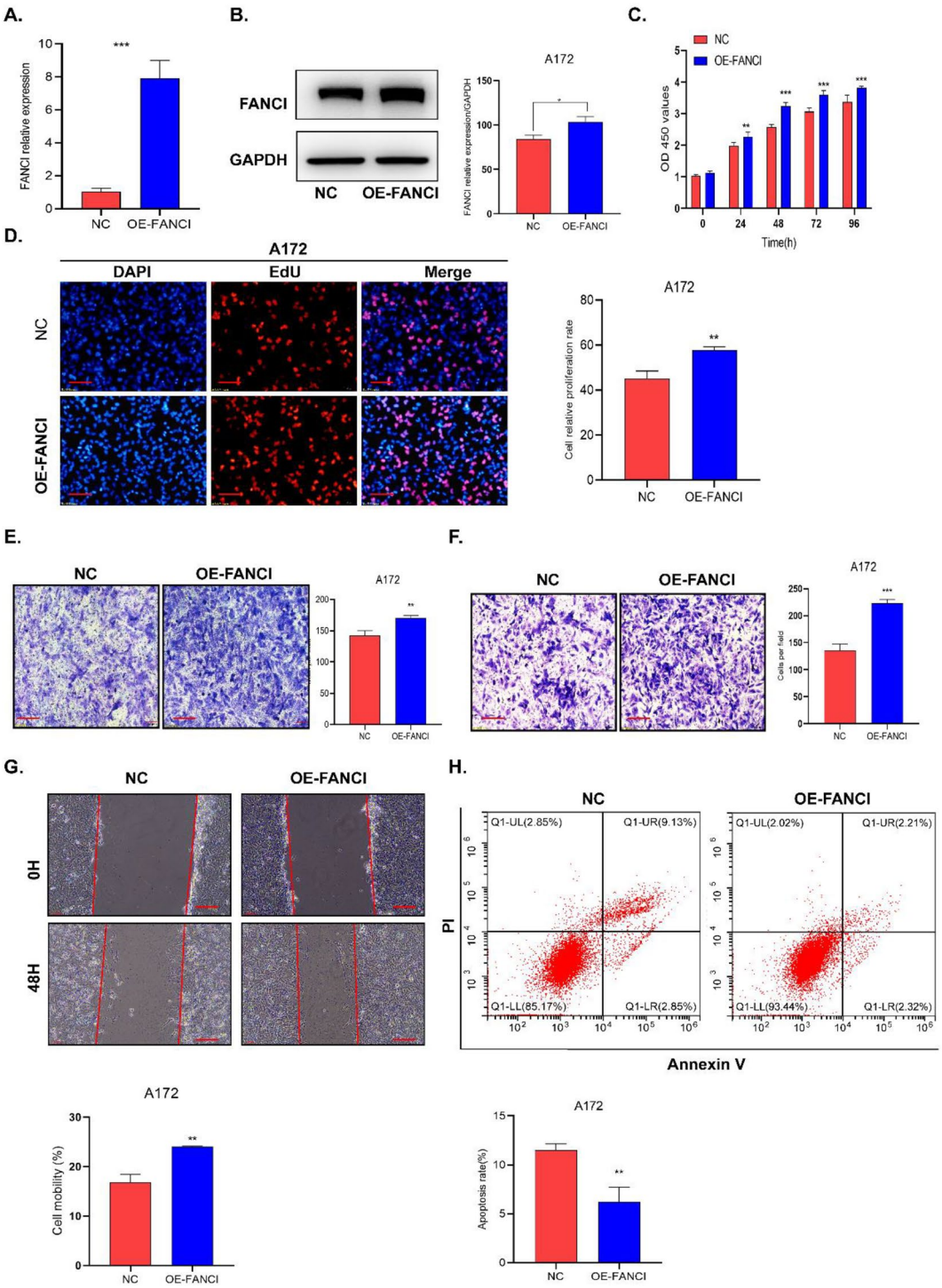

Fig. 4 Effect of FANCI overexpression on the biological behavior of A172 cells. **A**, **B** qRT-PCR and western blotting were used to verify the transfection effect of FANCI overexpression plasmid in A172 cells (OE-FANCI). **C**, **D** CCK-8 assay and EdU proliferation assay verified the effect of FANCI overexpression on the proliferation ability of A172 cells (scale bar = 100 μm). **E** Transwell (Matrigel-included) invasion assay to analyze the effect of FANCI overexpression on the motility of A172 cells (scale bar = 100 μm). **F-G** Transwell assay and cell scratch assay to analyze the effect of overexpression of FANCI on the migration ability of A172 cells (scale bar = 100 μm). **H** Apoptosis assay was to detect the changes in the apoptosis ability of A172 cells after overexpression of FANCI. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

D), invasion ability (Fig. 4E), migration (Fig. 4F, G), and inhibited apoptosis (Fig. 4H) of glioma cells. Taken together, our results point to FANCI expression being a key point for glioma cell growth.

2.4 FANCI mediate malignant progression by regulating the Akt/Bcl-2 pathway

In order to preliminarily explore the mechanism of FANCI's involvement in malignant progression, we further used the TCGA database to perform gene enrichment analysis on FANCI through the BEST website, and the results showed that FANCI may regulate the PI3K/Akt signaling pathway (Fig. 5A). We speculate that FANCI may regulate the malignant progression process of glioma cells through the PI3K/Akt pathway. In addition, We know from reading the literature that b-cell lymphoma-2 (Bcl-2) is an anti-apoptotic protein factor with the physiological function of inhibiting apoptosis and prolonging cell survival. Secondly, Bcl-2 is also one of the substrates of Akt. The activation of Akt phosphorylates Bcl-2, which enhances the anti-apoptotic effect of Bcl-2 [18, 19]. Interestingly, our results also showed that knockdown of FANCI in LN229 and U251 cells inhibited the expression of path-related proteins phosphorylated Akt (P-Akt ser473) and Bcl-2, and promoted the expression of the protein Bax (Fig. 5B). These results tentative suggested that there may be a regulatory mechanism between Akt and Bcl-2.

2.5 Knockdown of FANCI inhibits the tumorigenesis of glioma in vivo

Based on our above findings, lentivirus-transfected U251 cells were subcutaneously injected into nude mice as the control group (CON) and knockdown group (KD-FANCI) to establish a subcutaneous tumor model in nude mice to verify the effect of FANCI on glioma in vivo. The results showed that the size of Xenogeneic implant tumor formed by nude mice of KD-FANCI group was significantly smaller than that of the control group (Fig. 6A, B), and the weight of the tumors was also lower than that of the control group significantly (Fig. 6C). This is consistent with our previous results that FANCI promoted glioma growth. The glioma model was identified by HE staining, Ki-67 immunohistochemistry and glioma professor in the group (Fig. 6D, E). The qRT-PCR technology and Western blot technology verified the expression of FANCI in subcutaneous

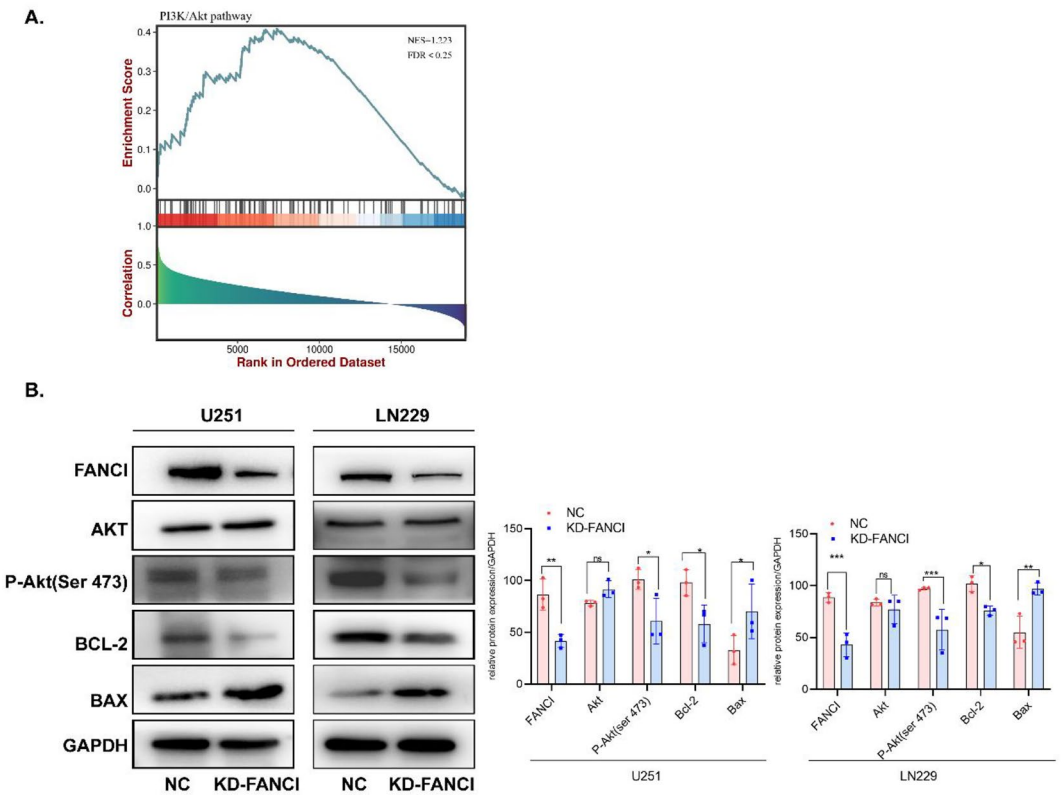

Fig. 5 FANCI expression and signaling pathways. **A** Gene enrichment analysis suggested that FANCI may regulate PI3K/Akt signaling pathway **B** FANCI low expression inhibited Akt phosphorylation (Ser 473) and Bcl2 expression. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

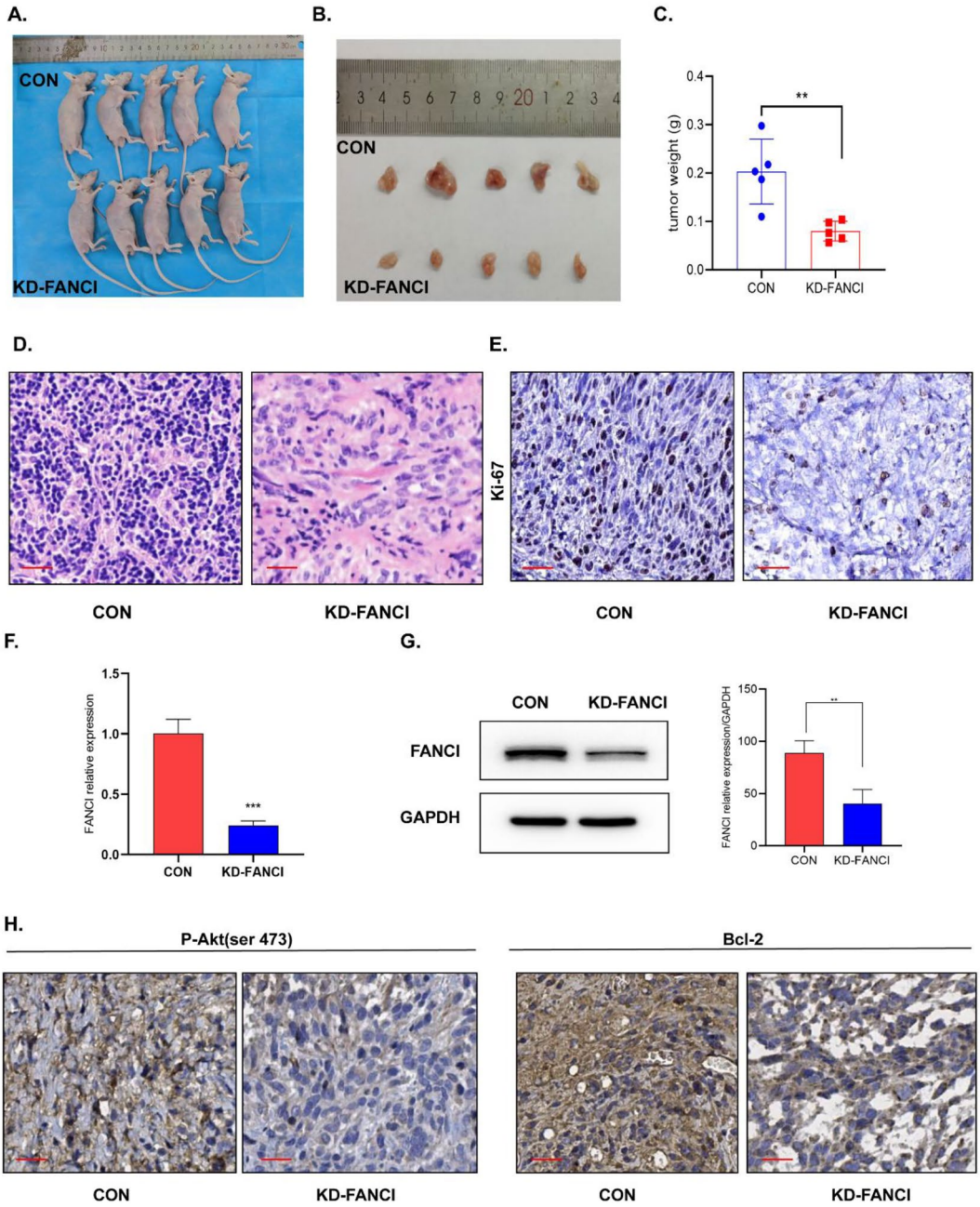

Fig. 6 Low level of FANCI expression inhibits tumorigenesis in nude mice. **A–C** Image of five nude mice in the CON/KD-FANCI group with size and weight of tumor differences. **D** HE staining. (scale bar = 200μm) **E** Immunohistochemistry to detect the expression of Ki-67 in nude mouse tumor tissues. **F**, **G** qRT-PCR and western blotting to verify the expression of FANCI in nude mouse tumor tissues. **H** Immunohistochemistry was used to detect the expression of P-Akt and Bcl-2. (scale bar = 200μm) $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

neoplastic tissue, and the results confirmed that the expression of FANCI in the KD-FANCI group was lower than that in the CON group (Fig. 6F, G). Immunohistochemistry (Fig. 6H) show that knockdown of FANCI expression regulates the expression of the P-Akt and Bcl-2. It seemed to validate our preliminary suspicion that there may be a potential regulatory mechanism between FANCI, Akt and Bcl-2.

3 Discussion

One cause of cancer is endogenous DNA replication mutations or exogenous DNA damage. However, some specialists suggest that exogenous factors may account for the majority [20]. But in any case, genomic instability is one of the bases of cancer development, which can accelerate the acquisition of genetic diversity and the occurrence of inflammatory responses in organisms [21]. FA is a gene-disease known for chromosomal instability, and evidence is accumulating that a wide variety of cancers with different FA pathway defects. The FA pathway may be helpful to hyperresponsive to cross-linkers to obtain more effective therapy by interference with it [22]. Therefore, as one of the key proteins in the FA pathway, FANCI has important practical significance in the occurrence and development of tumor. In recent years, many studies have found that silencing FANCI expression can inhibit the occurrence and development of cancers, such as ovarian cancer and lung adenocarcinoma, and also enhance the chemotherapy sensitivity of ovarian cancer [10, 23]. However, few studies have been reported on the expression of FANCI in glioma tissues, its relationship with prognosis, and the regulation of glioma progression.

Based on the sequencing data of our group, the results suggest that FANCI is a gene significantly upregulated in GBM tissues. Therefore, in this study, we further combined the analysis of different grades of glioma tissue chips and public databases, and we found that the expression of FANCI in human glioma tissues was increased, and the expression of WHO grade IV was significantly higher than that of other grades. Survival analysis also showed that FANCI expression was negatively correlated with overall survival and disease-free survival of glioma patients. These results suggest that FANCI may be involved in the growth of glioma and related to the prognosis of patients. FANCI expression and clinicopathological differences showed that patients aged ≥ 40 years had higher FANCI expression and higher Ki-67 expression. This may be related to the fact that the incidence of glioma changes with age, and glioblastomas tend to occur in middle-aged and elderly people [24]. In addition, it has been reported that Ki-67 can independently predict the prognosis of glioma [25], which is also consistent with the results of bioinformatics analysis obtained by us previously. Taken together, these results provide a theoretical basis for our following in vitro and in vivo experiments.

In this study, We found differences in the expression of FANCI in glioma cells, which we hypothesized might be related to the high heterogeneity of gliomas, including cell type and molecular characteristics. Secondly, there may be regulatory mechanisms such as DNA methylation that led to differences in FANCI expression levels. However, this speculation needs to be verified by further experiments in our follow-up work. In addition, we found that knockdown of FANCI expression inhibited the proliferation, migration, and invasion of glioma cells and promoted apoptosis, and vice versa. Subcutaneous tumor formation in nude mice indicated that knockdown of FANCI reduced the size and volume of subcutaneous gliomas in nude mice. Immunohistochemical staining also showed that in subcutaneous tumor tissues with low FANCI expression, the expression of the proliferative protein Ki-67 was lower than in high FANCI. This is consistent with previous findings that FANCI promotes lung cancer progression [26]. Our results indicated that FANCI was highly expressed in gliomas and acted as an oncogene.

Therefore, it is very important to further explore the mechanism of FANCI's involvement in glioma progression. The results of WB showed that the low expression of FANCI not only down-regulated the expression of phosphorylated Akt and Bcl-2 proteins, but also up-regulated the protein expression level of Bax. It indicating that FANCI may be involved in the malignant progression of glioma by regulating the Akt-Bcl-2. Although this is different from previous studies in which FANCI deletion led to an increase in Akt phosphorylation [19], we speculate that, firstly, due to the complexity of tumor types, experimental conditions, and the Akt pathway itself, the effect of FANCI on Akt activation may be different under different conditions. Second, the combination of our in vivo and in vitro experimental results and bioinformatics analysis showed that FANCI is a highly expressed oncogene in gliomas, and the activation of the PI3K/Akt signaling pathway is closely related to the growth and metabolism of gliomas [27], which suggests that inhibition of the expression of FANCI may leads to the reduction of Akt phosphorylation, which is in line with our findings. However, the mechanism of FANCI's regulation of the Akt pathway needs to be further explored by more experiments in future studies.

The PI3K-Akt signaling pathway is one of the classic activation pathways in cancer, which controls cell proliferation, cell metabolism, cell motility, apoptosis, and genomic instability [28, 29]. And the PI3K/Akt is also involved in the mechanism of cell drug resistance [6]. Moreover, Akt kinase is a key effector molecule of this pathway and is implicated in DNA damage [30]. One of the mechanisms by which cells survive is that Akt inhibits pro-apoptotic proteins and apoptotic processes, such as the anti-apoptotic protein Bcl-2 [31]. There is a strong link between Akt activation and

glioma prognosis, with a retrospective study showing elevated p-Akt (phosphorylation of Akt) expression in more than half of patients with high-grade glioma and a declining trend in overall survival [32]. According to the literature, phosphorylation of Ser 473 residues is necessary for the complete activation of Akt [31].

In addition to Bcl-2, mammalian target of rapamycin (mTOR) is also a common downstream effector of Akt, which is mainly involved in the regulation of cellular anabolic processes [33]. Although there are few studies on the regulation of mTOR by FANCI, due to the importance of FANCI in ribosome biosynthesis, there may be indirect pathways that regulate mTOR activity. For example, the mitogen-activated protein kinase (MAPK) pathway [34], which is involved in the regulation of cell proliferation, apoptosis and other physiological processes, is closely related to the mTOR pathway, and together they regulate cell growth, which is a potential therapeutic target for tumor progression. In addition, both Akt and MAPK are important components of cell signaling networks, and there is a mutual regulatory relationship between them. In addition, it has been reported [10] that FANCI can affect the activation of MEK/ERK/MMRs pathway by directly binding to inosine monophosphate dehydrogenase type II (IMPDH2), thereby promoting the migration and proliferation of lung adenocarcinoma cells. It is worth mentioning that PI3K/Akt and Wnt/β-catenin have been proposed as major targets for glioma drug design. The Wnt/β-catenin pathway is not only an important pathway affecting the tumor cell cycle, but also has a signaling cascade reaction with PI3K/Akt, which is a potential therapeutic measure for overcoming radio-resistance in gliomas [27]. In addition, targeting FANCI has been reported to increase breast cancer sensitivity to drugs, a promising therapeutic strategy [35]. Therefore, the application of FANCI as a tumor therapeutic target is still very promising and requires us to perform experimental projects such as Co-Immunoprecipitation and mass spectrometry to further explore how FANCI regulates the Akt pathway.

In summary, our findings suggest that FANCI has a carcinogenic role in glioma and there may be a potential regulatory mechanism between FANCI, Akt and Bcl-2. However, the mechanism of action of FANCI in the Akt/Bcl2 pathway and FANCI as a combination therapy regimen for PI3K/Akt signaling pathway in the treatment of glioma needs to be further verified and explored.

This study also has limitations. Our studies on FANCI promotion of glioma progression are still at a superficial stage. Since FANCI also plays an important role in DNA interstrand repair, yet there are no studies directly exploring cellular DNA damage and cell death as a result of suppressed FANCI expression, we need to design DNA damage experiments to explore the interaction between FANCI and DNA repair proteins in the future. Second, since Bcl-2 is regulated by a variety of factors, although we have seen a lot of literature pointing out that Akt regulates Bcl-2 expression, and the results of the present experiments have shown that phosphorylated Akt is consistent with the trend of Bcl-2 protein expression. However, we still need to design detailed experimental protocols in follow-up experiments to demonstrate that Akt regulation of Bcl-2 expression is involved in glioma progression, providing strong support for our initial speculation.

Disclosure The authors declare no ownership or commercial interest in any of the products mentioned or any of the concepts discussed in this article. All experiments involving human participants in this study were approved by Tianjin Huanhu Hospital (Institutional Review Board, Tianjin, China: CK-19-190,318), following the guidelines of the Declaration of Helsinki, and all patients provided written informed consent. All animal experiments were conducted in accordance with the standards of the Guidelines for the Care and Use of Laboratory Animals (Ethics No. 2021C180) approved by the Animal Ethics Committee of Jining Medical College, and the maximum diameter of the tumors in this experiment did not exceed 15 mm, which was in accordance with ethical standards.

Author contributions The idea of this study was proposed by SJK. LHY and LYR wrote this article, LHY, LJL and QXY was responsible for the experimental operation. WMY and XSS conducted the animal experiment part and figures. LHY, LYR, and LBB prepared grammar revisions. SJK, JF and LY revised the final draft. All authors have read and agree to publish the final manuscript.

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Data availability All data generated or analyzed during this study are included in this published article.

Declarations

Competing interests The authors declare no competing interests.

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