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Research article

Knockdown of FANCI suppresses hepatocellular carcinoma development via the PI3K/Akt/GSK-3β pathway

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ABSTRACT

Background: Abnormal expression of Fanconi anaemia complementation group I (FANCI) has been implicated in carcinogenesis. However, the precise role of FANCI in the development of hepatocellular carcinoma (HCC) remains unclear.

Materials & methods: We conducted a comprehensive bioinformatics analysis of FANCI's role based on HCC patient sequencing data in the TCGA and GEO databases. Then, we performed qPCR, Western blotting (WB), and immunohistochemistry (IHC) assays. SiRNA-mediated knockdown of FANCI was conducted, followed by CCK-8, EdU staining, and colony formation experiments to evaluate the impact of FANCI knockdown on HCC cell behaviour. Flow cytometry was employed to explore alterations in the cell cycle after FANCI knockdown in HCC cell lines. Furthermore, RNA sequencing was performed to investigate potential mechanisms following FANCI knockdown, and WB analysis was used to validate the corresponding pathway.

Results: Our bioinformatics analysis revealed elevated expression of FANCI in HCC, which was subsequently validated through qPCR, WB, and IHC assays. High expression of FANCI was significantly associated with a poor prognosis in HCC patients. Univariate and multivariate Cox regression analyses identified FANCI as an independent prognostic risk factor for HCC patients. Additionally, the coexpressed genes of FANCI were found to be associated with multiple cancer pathways. Knockdown of FANCI expression significantly inhibited HCC cell proliferation and colony formation by inducing cell cycle arrest. Further WB analysis revealed that FANCI knockdown suppressed the expression of Cyclin D1 and p-AKT while increasing the expression of GSK-3β in HCC cells. However, no significant differences were observed in the expression levels of AKT and PI3K.

Conclusion: Overall, our research provides substantial proof of FANCI's crucial function as an oncogene in HCC. It could serve as a potential prognostic marker, therapeutic target, and tumorigenic factor in HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is responsible for 75–85 % of liver cancer-related deaths, and it is the sixth most common cancer worldwide. With approximately 906,000 new cases and 830,000 registered deaths each year, HCC ranks as the third leading cause of

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cancer-related mortality globally [1,2]. Risk factors for developing HCC include hepatitis virus infection, excessive alcohol intake, and metabolic conditions [3]. Despite the availability of various treatment options, such as surgery, chemotherapy, radiation, targeted therapy, and immunotherapy, the mortality and recurrence rates for HCC remain high [4]. The underlying mechanisms of HCC are not yet fully understood, emphasizing the urgent need to identify more effective markers for guiding the development of novel therapies.

The Fanconi anaemia (FA) protein family, consisting of 15 members involved in DNA damage repair and cell cycle control, includes Fanconi anaemia complementation group I (FANCI) [5]. Previous research has established connections between FA proteins and the development of various malignancies. For example, a defect in Fanconi anaemia complementation group D2 (FANCD2) has been associated with skin cancer [6], while inhibiting Fanconi anaemia complementation group F (FANCF) has been found to prevent doxorubicin resistance in ovarian cancer cells [7]. Furthermore, FANCI knockdown increases DNA damage-induced apoptosis in ovarian cancer [8]. FANCI has also been implicated in colon cancer carcinogenesis and breast cancer susceptibility [9,10]. However, the exact role of FANCI in HCC remains to be fully elucidated.

Our study aims to explore the potential role of FANCI in HCC using a comprehensive approach that integrates multiple platforms, databases, and bioinformatics techniques. Additionally, we will assess the biological effects of FANCI knockdown in HCC cell lines to better understand its function.

2. Materials and methods

2.1. Data analysis

Transcriptome data for HCC samples (374 tumour tissues and 50 adjacent normal tissues) and corresponding clinical data were obtained from The Cancer Genome Atlas (TCGA) database (https://cancergenome.nih.gov). Gene expression data from GSE101685 (8 normal tissues and 24 HCC samples), GSE101728 (7 pairs of HCC and matched adjacent tumour-free tissues), and GSE121248 (69 HCC samples and 55 matched adjacent normal tissues) were retrieved from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/). The expression of FANCI in different cancer types was assessed using the Tumour Immune Estimation Resource (TIMER) database (https://cistrome.shinyapps.io/timer/) [11]. Overall survival analysis HCC patients with high vs. low FANCI expression was conducted using the Gene Expression Profiling Interactive Analysis 2 (GEPIA2) database (http://gepia.cancer-pku.cn) [12]. Coexpressed genes were obtained from the LinkedOmics database (http://linkedomics.org/login.php) [13].

2.2. Patient & tissue samples

The study was approved by the Guangzhou First People's Hospital Ethics Committee, and written consent was obtained from all participants (K-2018-142-02). Real-time fluorescent quantitative polymerase chain reaction (qRT−PCR) was performed on 9 pairs of samples to determine the transcriptional expression of FANCI. Western blot analysis was conducted on a total of 12 pairs of samples from HCC patients at Guangzhou First People's Hospital.

2.3. qRT−PCR

RNA was extracted from cells or homogenized material using TRIzol (Thermo Fisher Scientific, USA). Reverse transcription was performed using the RT−qPCR kit with gDNA remover (MIKX, China). Fluorescence measurement was carried out using the Roche Light Cycler 480 with the SYBR technique (LC480). Statistical analysis was performed using the *t*-test. The primer sequences used were as follows:

FANCI, F:5′- CCACCTTTGGTCTATCAGCTTC-3′, R:5′- CAACATCCAATAGCTCGTCACC-3'; GAPDH, F:5′-CTGGGCTA-CACTGAGCACC-3′, R:5′- AAGTGGTCGTTGAGGGCAATG -3'.

2.4. Western blotting

Proteins were separated by 8 % sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and transferred to a polyvinylidene fluoride membrane (Merck Millipore, IPVH00020, USA). The membrane was blocked with QuickBlock Western Sealing Fluid (P0252-500, China) and incubated with primary antibodies overnight at 4 °C. After washing with TBST, the membrane was incubated with secondary antibodies at room temperature. Chemiluminescence imaging equipment and a modified chem-iluminescence kit (Bio-Rad, UA, USA) were used for band detection. The antibodies used were as follows:

anti-FANCI antibody (Abcam, ab245219, USA), anti-Cyclin D1 antibody (Abcam, ab134175, USA), PI3K/AKT signalling pathway (Abcam, ab283852, USA), and anti-alpha tubulin polyclonal antibody (Elabscience, E-AB-20069, China).

2.5. Cell culture

The HCC cell lines SMMC-7721 and Hep-G2 were obtained from the Chinese Academy of Sciences' Type Culture Collection and maintained in DMEM Basic (1X) medium (Gibco, USA) supplemented with 10 % foetal bovine serum and 1 % penicillin−streptomycin. The cells were incubated at 37 °C with 5 % CO2. SMMC-7721 and Hep-G2 cell were tested and authenticated by STR at the cell bank, the cells were subsequently cultured on a massive scale after shipping and were stored at −80 °C.

2.6. siRNA transfection

Cells were seeded in six-well plates and transfected with FANCI-specific siRNA oligos (GenePharma, China) using LipofectamineTM RNAiMAX Reagent (Thermo Fisher, USA) according to the manufacturer's instructions. The transfected cells were incubated with the siRNA for 8 h in serum-free medium, followed by replacement with complete culture medium. The siRNA sequences were as follows.

FANCI-siRNA#1:5′-CCUGAGAGCCAUCUUCAAATT-3';
FANCI-siRNA#2:5′- GCAGGUGGGAUCAGCAAUATT-3';
FANCI-siRNA#3:5′GCACCAUUAUUCUACACAUTT -3';
siRNA-NC: 5′- UUCUCCGAACGUGUCACGUTT-3'.

2.7. Cell Counting Kit-8 (CCK-8) assay

Hep-G2 and SMMC-7721 cells were seeded in 96-well plates and transfected with FANCI siRNA. After 1 h of coculture with Cell Counting Kit-8 reagent (Dojindo, Japan), cell viability was measured using a microplate reader at 450 nm. Viability readings were taken over five days.

2.8. Colony formation assay

SMMC-7721 and HepG2 cells were transfected with siRNA and seeded in six-well plates. The culture medium was changed every three days to allow colony formation. After fixing the samples with 4 % formaldehyde and staining with Coomassie brilliant blue, the number of colonies was counted.

2.9. EdU (5-Ethynyl-2′- deoxyuridine) assay

SMMC-7721 and Hep-G2 cells were transfected with siRNA for 72 h and then cultured with 1 % EdU for 4 h using the Cell-Light EdU Apollo 567 In Vitro Kit (Guangzhou, China). The cells were fixed with 4 % formaldehyde and stained with DAPI and Apollo. Images were captured using a laser microscope.

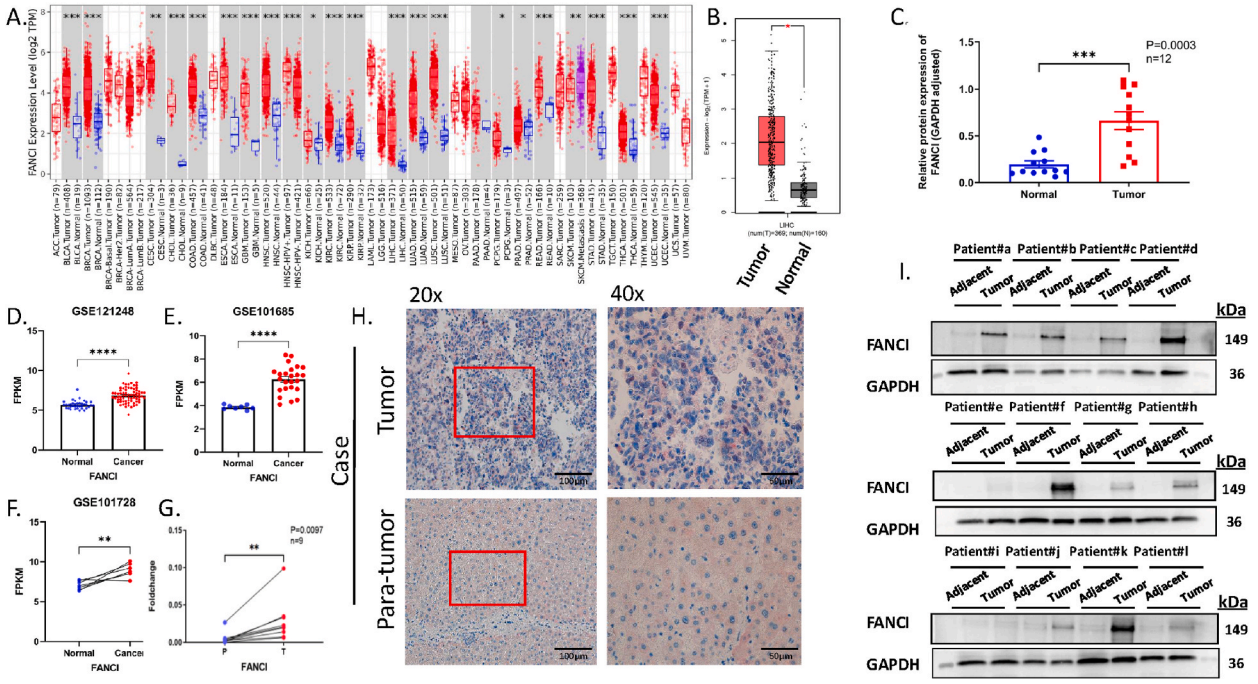

Fig. 1. FANCI is highly expressed in HCC.
(**A**) Differential expression of FANCI in different tumours based on the TIMER database. (**B**) Comparison of FANCI expression between HCC and normal tissues based on GEPIA2. (**C**) Western blot detection with statistical analysis for samples from 12 patients. (**D, E, F**) Validation of differential FANCI expression in GSE101685, GSE101728 and GSE121248. (**G**) mRNA expression level of FANCI in 9 paired HCC tissues and adjacent normal tissues detected by qPCR. (**H**) Representative images of immunohistochemical staining for FANCI expression differences in HCC tissues and paired adjacent normal tissues. (**I**) Protein expression level of FANCI in 12 paired HCC tissues and para-carcinoma tissue detected by Western blotting (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.The original figures are in Supplementary material 1 original blots-Fig. 1I).

2.10. Flow analysis of the cell cycle

HepG2 and SMMC-7721 cells transfected with siRNA were collected, fixed with 75 % ethanol, and incubated with PI reagent (Annexin V/PI apoptosis kit, Cat# AP101, China). Flow cytometry analysis was performed to analyse the cell cycle distribution using FlowJo software.

2.11. Statistical analysis

Statistical analysis was performed using the *t*-test, Wilcoxon signed rank test, or Kruskal–Wallis H rank sum test, depending on the

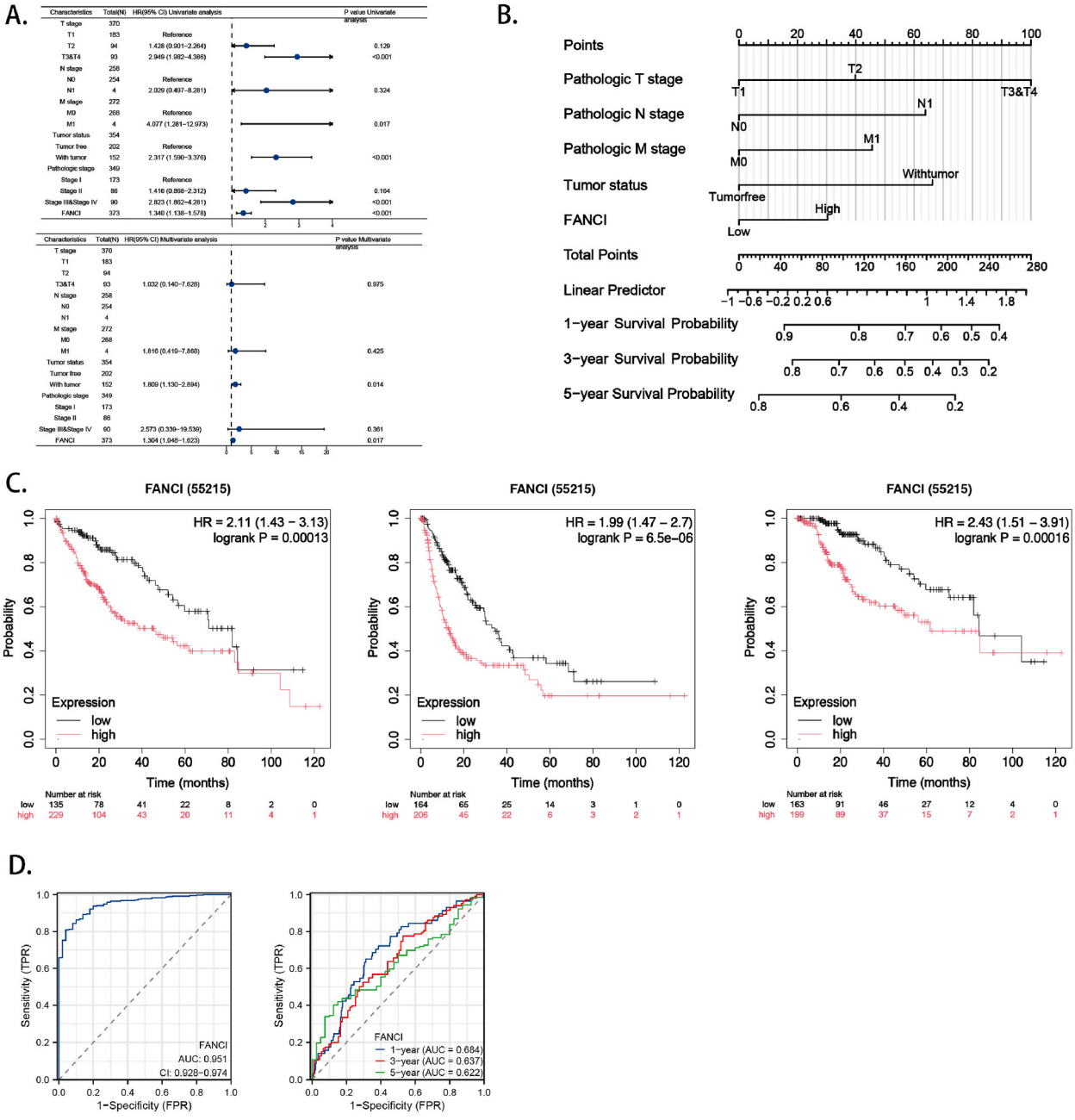

Fig. 2. Diagnostic value of FANCI in HCC patients.
(**A**) Univariate and multivariate Cox regression analyses of prognostic-related risk factors. (**B**) Nomogram map showing FANCI predictive efficiency. (**C**) High expression of FANCI was correlated with poor OS, PFI, and DSS in HCC. (**D**) Diagnostic efficacy curve of FANCI in HCC. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. OS, overall survival; PFS, progression-free survival; DSS, disease-specific survival; AUC, area under the curve.

data distribution. The significance levels were corrected using the Bonferroni or Dunnett method. Differences were considered statistically significant at $P < 0.05$ (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

3. Results

3.1. The expression level of FANCI in both normal and tumour tissues

To assess FANCI expression in normal and tumour tissues, we conducted an analysis using the TIMER2 database. Our results indicated that FANCI expression was significantly higher in 21 different types of tumours than in adjacent normal tissues (Fig. 1A). Due to the limited number of normal samples, we employed the GEPIA2 database and acquired normal tissue expression data using the GTEx database from GEPIA2; there were significant differences in expression between tissue types (Fig. 1B). To further validate our findings, we conducted analyses using three additional independent GEO datasets specific to LIHC: GSE101685, GSE101728, and GSE121248. The analysis of these datasets confirmed the elevated expression of FANCI in tumour tissues compared to adjacent normal tissues, supporting the consistency of our results across different datasets (Fig. 1D–F).

To further validate the expression of FANCI in hepatocellular carcinoma (HCC), we obtained HCC and adjacent normal tissue samples from Guangzhou First People's Hospital. We performed analyses on nine paired HCC patient samples at the transcript level (Fig. 1G, $P = 0.0097$, $n = 9$) and twelve paired samples at the protein level (Fig. 1C–I, $P = 0.0003$, $n = 12$). The results from both analyses confirmed that FANCI expression was significantly higher in tumour tissues than in adjacent normal tissues. This finding was further supported by immunohistochemical analysis of HCC patient tissue (Fig. 1H, $P = 0.0097$, $n = 9$), which showed that FANCI is overexpressed in HCC.

3.2. The diagnostic value of FANCI in HCC patients

Subsequently, we investigated the association between FANCI expression and the prognosis of HCC patients. The HCC patients' baseline data are shown in Supplemental Table 1. The correlation between FANCI expression and the clinical characteristics of HCC patients was analysed by Cox regression. The univariate Cox analysis revealed that stage T3 & T4 (HR = 2.949, $P < 0.001$), stage M1 (HR = 4.077, $P = 0.017$), stage III & IV (HR = 2.823, $P < 0.001$), and FANCI expression (HR = 1.340, $P < 0.001$) were potential high-

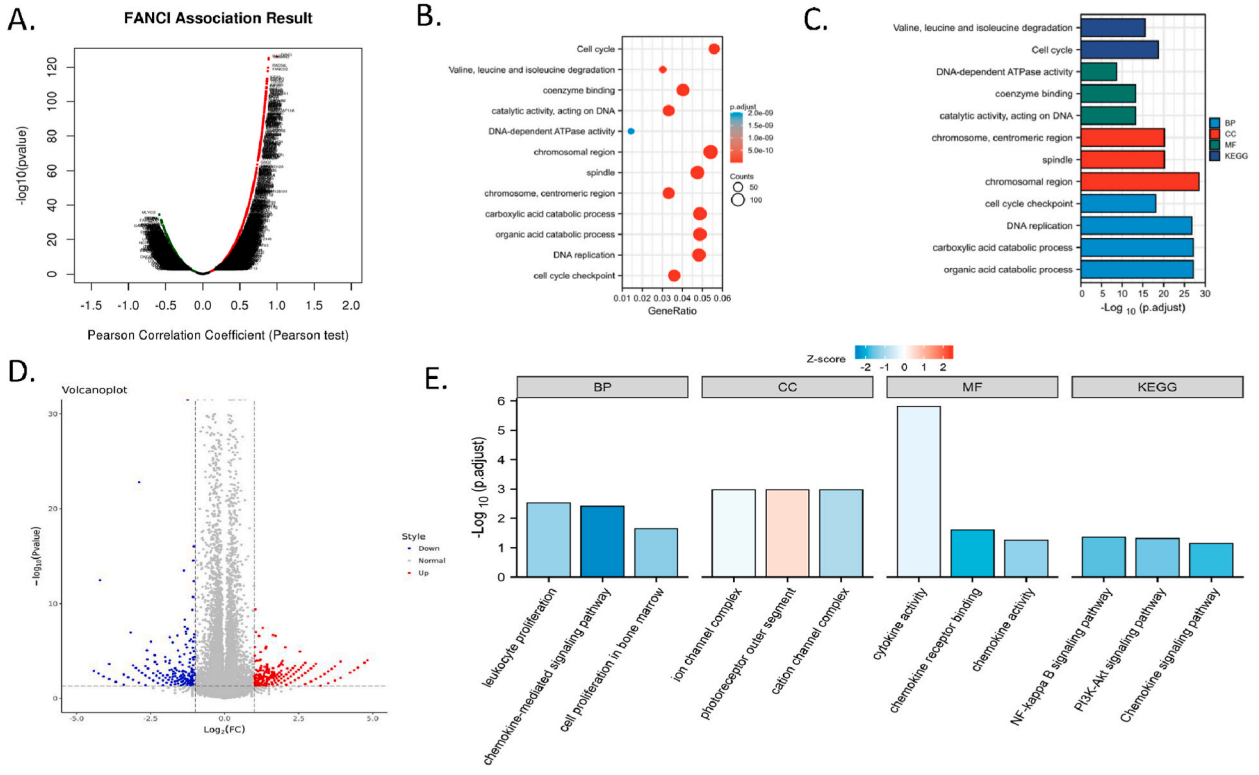

Fig. 3. Coexpressed genes of FANCI and GO/KEGG analysis.
(**A**) Volcano plot based on coexpressed genes of FANCI from the LinkedOmics database. (**B, C**) Bar chart and bubble chart of significantly enriched GO/KEGG pathway analysis based on coexpressed genes. (**D**) Volcano map of the differentially expressed genes between the si-FANCI#3 group and the si-NC group. (**E**) GO and KEGG analysis of differentially expressed genes from the RNA sequencing analysis.

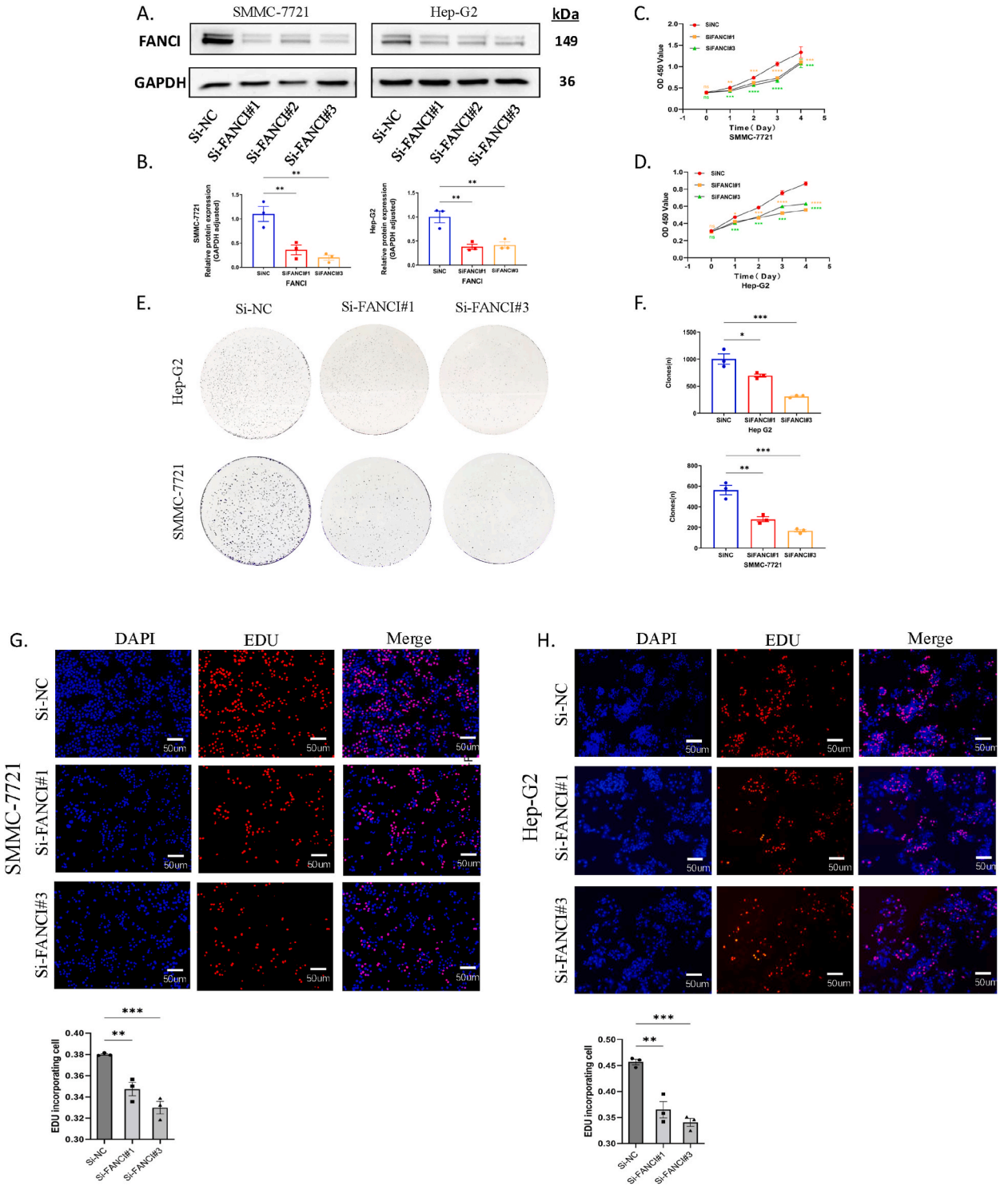

Fig. 4. Knockdown of FANCI inhibits the proliferation of HCC cell lines.
(**A, B**) Efficiency of FANCI-siRNA transfection at the protein level and statistical analysis (The original figures are in Supplementary material 2 original blots-Fig. 4A). (**C, D**) Knocking down FANCI inhibits the proliferation of the HCC cell line (as indicated by CCK-8 assay). (**E, F**) Colony formation results after FANCI-siRNA transfection in HCC cell lines and statistical analysis. (**G, H**) Representative diagram of EdU SMMC-7721 cells (left) and HepG2 cells (right) after transfection of si-NC, si-FANCI#1 and si-FANCI#3 and statistical analysis.

risk factors for HCC. These variables were further investigated through multivariate analysis, which revealed that tumour status (HR = 1.809, P = 0.014) and FANCI expression (HR = 1.304, P = 0.017) were independent risk factors for HCC patients (Fig. 2A). In addition, a nomogram was constructed for predicting the probability of 1-,3-,5-year overall survival for HCC patients (Fig. 2); the C-index values of the nomograms were as follows: OS nomogram, 0.663 (0.625–0.700); DSS nomogram, 0.854 (0.832–0.875); and PFI nomogram, 0.795 (0.776–0.814), indicating the model had a good performance in predicting (Fig. 2B). The diagnostic efficiency of FANCI was evaluated through ROC analysis and demonstrated excellent performance, with AUC values of 0.951, 0.684 (1 year), 0.637 (3 years), and 0.622 (5 years) (Fig. 2D).

3.3. Biological functions potentially regulated by FANCI

To investigate the potential biological role of FANCI in hepatocellular carcinoma (HCC), we analysed its coexpressed genes using the LinkedOmics database. A total of 1885 coupregulated and 913 codownregulated genes were identified, as demonstrated in the volcano map (Fig. 3A). Gene set enrichment analysis (GSEA) was then performed to examine enriched Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways based on the coexpressed genes. The results indicated that FANCI was correlated with DNA replication, the cell cycle, the cell cycle checkpoint, and DNA-dependent ATPase activity (Fig. 3B–C). We performed RNA sequencing on three pairs of SMMC-7721 cells transfected with si-FANCI#3 (for FANCI knockdown) and si-NC (a control) for further validation, and the differentially expressed genes are depicted in a heatmap and a volcano plot (Fig. 3D). GO/KEGG analysis revealed that these genes were mostly linked to the PI3K-Akt signalling pathway, bone marrow cell proliferation, and leukocyte proliferation (Fig. 3E). Based on the results of comprehensive bioinformatics analysis and RNA sequencing of cells with and without FANCI knockdown, the cell cycle and PI3K-Akt signalling pathway may be involved in FANCI's role in HCC.

3.4. Hepatocarcinoma cell proliferation was inhibited after knockdown of FANCI

To investigate the contribution of FANCI to hepatocellular carcinoma (HCC) development, FANCI was silenced in SMMC-7721 and Hep-G2 cell lines using siRNA. The knockdown efficiency of FANCI protein was evaluated by Western blotting (Fig. 4A and B). The proliferation of HCC cell lines was significantly reduced upon FANCI silencing, as demonstrated by CCK-8 experiments (Fig. 4C and D) and confirmed by colony formation assays (Fig. 4E and F). According to EdU staining, siRNA-transfected HCC cells showed a lower percentage of cells double-stained with EdU and DAPI than the si-NC group (Fig. 4G and H).

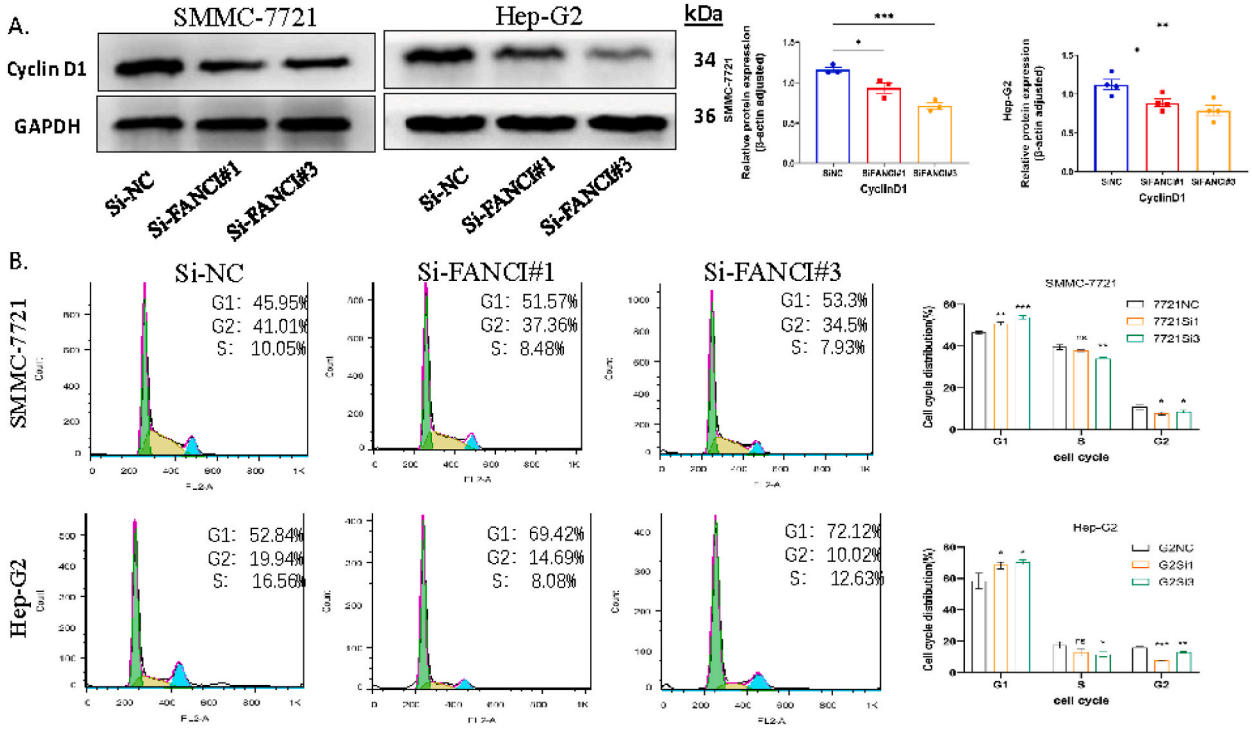

Fig. 5. Knockdown of FANCI interferes with the cell cycle.
(**A**) Expression of cyclin D1 after knockdown of FANCI in two HCC cell lines and statistical analysis (The original figures are in Supplementary material 3 original blots-Fig. 5A). (**B**) Flow cytometry analysis of HCC cell lines after transfection of si-NC, si-FANCI#1 and si-FANCI#3 and matched statistical analysis.

3.5. Cell cycle progression was inhibited after knockdown of FANCI in HCC

We used flow cytometry to further explore the potential mechanism after silencing FANCI in HCC cell lines and found that the HepG2 and SMMC-7721 si-FANCI transfection groups had greater G1 phase ratios than the si-NC group (Fig. 5B). The expression of Cyclin D1, a key player in the G1-S phase transition of the cell cycle, was reduced upon FANCI silencing, as revealed by Western blot analysis (Fig. 5A).

3.6. FANCI knockdown interrupts HCC proliferation via the PI3K/Akt/GSK-3β pathway

Additionally, we examined the expression of related proteins in the si-FANCI and si-NC groups of these two HCC cell lines and discovered that transfection of FANCI-siRNA decreased p-AKT levels and increased GSK-3β levels in both SMMC-7721 and Hep-G2 cells, while no differences were seen in PI3K and Akt levels (Fig. 6).

After transfection with FANCI-siRNA, WB detection revealed that Hep-G2 and SMMC-7721 cells had different protein levels of PI3K, Akt, p-Akt, and GSK-3β (The original figures are in Supplementary material 4 original blots-Fig. 6).

4. Discussion

The FA protein family member FANCI, which interacts interdependently with the FANCD2 protein in response to replication stress, is an essential part of the FA pathway [14]. FANCI, which has a molecular weight of 149 kDa, is located in the nucleolus and is physically and functionally connected to the transcription of preribosomal RNA [15]. Previously, the aberrant expression of the FANCI protein in cervical cancer was linked to a poor prognosis in several patient cohorts, and prior research also links FANCI to the emergence of a number of malignancies [16]. The silencing of FANCI has been demonstrated to cause DNA damage and enhance susceptibility to the chemotherapeutic drug carboplatin in ovarian cancer [8]. By activating the MEK/ERK/MMP pathway, FANCI and IMPDH2 work together to enhance tumour development in lung adenocarcinoma [17]. Despite these results, more research is necessary to determine the specific function of FANCI in HCC.

In our study, FANCI was considered a potential biomarker and therapeutic target. Alpha-fetoprotein (AFP) is the first and most widely used serum marker in the detection of HCC and has been applied in clinical diagnosis for many years since the 1970s [18,19]. Although AFP positivity can reach 72–90 % in patients with HCC, the sensitivity is only 9–32 % [20], which also limits the accuracy of HCC diagnosis. Moreover, nearly 1/3 of patients with HCC exhibit normal serum levels of AFP [21]. Therefore, it is of great clinical significance to search for more diagnostic and prognostic biomarkers for HCC. Over the past few years, several biomarkers have been developed from sequencing and public databases. For example, des-γ-carboxy prothrombin (DCP) seems to have become a more appropriate biomarker for HCC detection due to its high specificity and better prognosis prediction ability for HCC [22,23]. A novel bioassay for the Sonic Hedgehog (SHh) ligand in tissue specimens may help diagnose HCC with negative AFP and predict early microvascular invasion [24]. Serum annexin A3 (ANXA3) provides greater diagnostic performance than AFP, especially in early diagnosis and discriminating HCC from patients at risk [25]. Some biomarkers, such as AFP, are only useful as markers of progression rather than targets for therapy, whereas for other biomarkers, such as HER2 in breast cancer [26], EGFR in lung cancer [27] and KRAS in colorectal cancer [28], can act as fundamental factors promoting tumour progression and deterioration. Thus, our study indicates that FANCI may not be the only biomarker to indicate the progression of HCC, and the results of functional experiments after knocking down FANCI implied that it could also be a potential therapeutic target for HCC.

A previous study mainly focused on FANCD2 rather than FANCI [29,30], and the role of FANCI in HCC development was not clear. Thus, by exploring FANCI as a potential HCC biomarker, we provided a new indicator for assessing the progression and treatment response of HCC and further revealed the potential mechanism of HCC occurrence and development. As FANCI is mainly involved in critical processes such as DNA damage repair and the cell cycle [31,32], our results also consistently found that knocking down FANCI

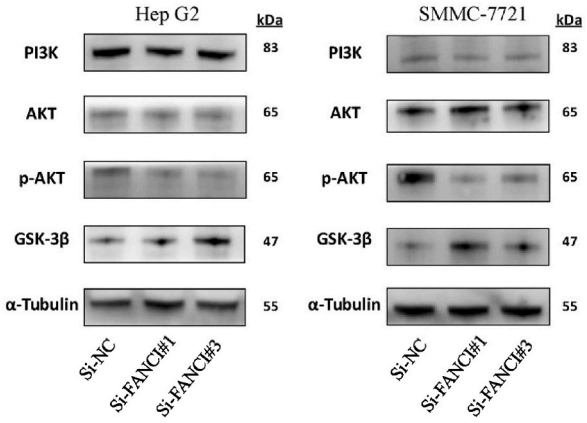

Fig. 6. Potential molecular mechanism of cell cycle arrest mediated by FANCI.

in hepatocellular carcinoma blocks the cell cycle. The abnormal expression or mutation of FANCI may lead to a decrease in DNA damage repair ability, thereby increasing the sensitivity of tumour cells to treatment, and abnormal expression of FANCI in HCC patients may result in drug resistance or improve drug sensitivity, which could further guide clinical treatment. Therefore, the exploration of FANCI as a therapeutic target provides a new view for developing new treatment strategies for HCC.

This study combined bioinformatics analysis and experimental validation to fully investigate the potential function of FANCI in HCC. Specifically, bioinformatics analysis can quickly identify potential biomarkers and therapeutic targets through screening and analysis of large-scale gene expression data. On the other hand, we performed experiments to validate the results of bioinformatics analysis and further investigate the specific effect and potential mechanism after knockdown of FANCI in HCC cell lines, strengthening the reliability and interpretability of this study. Our results indicated that FANCI may be related to the cell cycle and cell proliferation. A previous study found that FANCI associates with SF3B1 in chromatin to prevent the accumulation of postcatalytic intron lariats and contributes to the timely release of splicing factors [33]. We suspected that FANCI may combine with cell cycle-related proteins or proteins associated with cell proliferation and further exert its function in HCC development. Moreover, FANCI was located in the cell nucleus, so it is possible that FANCI binds to the mRNA of certain key genes involved in the cell cycle or cell proliferation, affecting their entry and exit into the nucleus. Therefore, we will search for the specific mechanism of FANCI in the future.

While our study has provided valuable insights, it is essential to acknowledge its limitations. First, we did not perform functional experiments based on FANCI overexpression, but we will conduct rescue experiments on FANCI-overexpressing cell models in the future to make the results more reliable. Second, verifying the function of FANCI in animal experiments is an essential part of our future work. We hope that this work will be helpful in clarifying the role of FANCI in HCC.

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5. Conclusion

Overall, our research provides substantial proof of FANCI's crucial function as an oncogene in HCC. It serves as a prognostic marker, a therapeutic target, and tumorigenic factor in HCC. Nonetheless, more research into the specific processes underlying FANCI in HCC is necessary.

CRediT authorship contribution statement

Ziwei Yin: Writing – original draft, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Minqiang Lu:** Writing – review & editing, Supervision, Resources, Conceptualization. **Rongdang Fu:** Writing – review & editing, Supervision, Project administration, Conceptualization.

Declaration of competing interest

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2025.e42731.

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