

Combined Inhibition of AURKA and HSF1 Suppresses the Cell Proliferation and Promotes Cell Apoptosis of Hepatocellular Carcinoma by Activating Endoplasmic Reticulum Stress

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Abstract

Objective: This study aims to investigate the anti-tumor effect of combined inhibition of aurora kinase A (AURKA) and heat shock transcription factor 1 (HSF1) for hepatocellular carcinoma (HCC) and the underlying mechanism.

Methods: The expression of AURKA and HSF1 in HCC tissues and cell lines were detected by Immunohistochemistry (IHC), qRT-PCR and Western blot. Then, AURKA was downregulated in HepG2 cells by lentivirus-induced RNA interfere. Cell viability, invasion, migration and apoptosis were examined by CCK8, clone formation, transwell assays and flow cytometry analysis. The expression of proteins related to cell cycle, apoptosis and endoplasmic reticulum stress (ERS) were detected by Western blot. The tumor in vivo growth was measured on nude mice model. Histopathological examination was performed with HE staining.

Results: AURKA and HSF1 were highly expressed in HCC tissues and cells, as well as negative related with prognosis. Knockdown of AURKA significantly inhibited the colony formation and migration of HCC cells. Besides, AURKA inhibitor (Danusertib) significantly reduced the proliferation and migration of HCC cells, and promoted cell apoptosis. Combined inhibition of AURKA and HSF1 induced cell apoptosis of HCC cells and increased the expression of ERS-associated proteins including p-eIF2 α , ATF4 and CHOP in vitro. Additionally, combined inhibition of AURKA and HSF1 displayed an excellent antitumor activity on HCC model with relative low cytotoxicity.

Conclusion: Combined inhibition of AURKA and HSF1 display excellent anti-tumor effect on HCC by activating endoplasmic reticulum stress, suggesting that AURKA and HSF1 can served as potential targets for HCC treatment.

1 Background

Liver cancer is one of the most common cancers in the world, and its incidence rate ranks sixth and its mortality rate ranks fourth worldwide[1]. Hepatocellular carcinoma (HCC) is the main type of liver cancer, accounting for about 75%-85% of cases[1]. HCC has the characteristics of high fatality rate, high metastasis and high invasiveness[2]. Although significant progress has been made in liver cancer surgical resection, liver transplantation, and drug therapy in recent years, the prognosis of HCC patients is still poor[3]. The pathogenesis of HCC involves multiple signaling pathways, and the molecular mechanism of its malignant progression is unclear. Therefore, it is of great clinical significance to find more effective early diagnosis markers and targeted therapy targets for tumors.

Aurora kinase A (AURKA) is a cell cycle regulating enzyme, which starts to locate in the S phase of mitosis, and increases rapidly in the late G2 and M phases until the G1 phase of the next cycle[4]. During the process of chromosome separation, AURKA participates in the formation of microtubules and/or the stability of the spindle pole, promotes the maturation of the centrosome, and ensures the smooth progress of the mitotic cell cycle[5]. At present, studies have reported that the expression of AURKA is

increased in many cancers, such as HCC, breast cancer, colorectal cancer, bladder cancer, and head and neck squamous cell carcinoma, etc., and is related to clinical staging, local lymph node metastasis and distant metastasis[6–11]. By activating a variety of signal pathways during mitosis, AURKA can accelerate the cell cycle process and promote the activation and metastasis of tumor cells[12]. Besides, AURKA can promote the occurrence and development of tumors by accelerating epithelial-mesenchymal transition and regulating tumor angiogenesis[13, 14]. Thus, AURKA may be served as an important target for the treatment of HCC tumors, and clarifying the effect of AURKA on HCC function and its mechanism will provide experimental evidence for the clinical use of AURKA as the target of HCC gene therapy, which has important clinical significance.

In addition, heat shock transcription factor 1 (HSF1), as an important transcriptional regulator, is essential for the normal development of the body, and the abnormal expression of HSF1 is closely related to the occurrence and severity of malignant tumors[15]. Studies have found that the expression and transcriptional activity of HSF1 in HCC is higher than that of normal liver tissues[16]. HSF1 regulates the expression of related genes and the growth and development of cancer cells by mediating the transcription of heat shock proteins[17]. At the same time, HSF1 can promote the drug resistance of HCC cells by regulating the level of autophagy[18]. Therefore, this study aimed to investigate the effect of co-inhibition of AURKA and HSF1 on HCC and explore the potential mechanism, thereby providing a new therapy for HCC.

2 Material And Methods

2.1 HCC specimens

Totally 3 pairs of HCC tissues and corresponding adjacent normal tissues were obtained from the Department of Pathology, Jinling Hospital, Medical School of Nanjing University. The patients did not receive any chemotherapy, immunotherapy or radiotherapy before collecting the specimens. This study was approved by the Ethics Committee of Jinling Hospital, and the written informed consent were obtained from patients.

2.2 Cell Culture

HepG2 cells were purchased from the Type Culture Collection of Chinese Academy of Sciences (Shanghai, China), and maintained in Roswell Park Memorial Institute (RPMI) -1640 medium (Gibco, Scotland, UK) containing 10% fetal bovine serum (FBS; Sigma, St. Louis, MO, USA), 100 U/ml penicillin, and 100 µg/ml streptomycin. All cells were cultured at 37 °C with 5% CO₂.

2.3 Transfection assay

Small-interfering RNA (siRNA) targeting AURKA and a negative control were purchased and synthesized by RIBOBIO (Suzhou, China). The target sequence of AURKA-siRNA was 5'-ATGCCCTGTCTTACTGTCA-3', and the siN05815122147 NControl_05815 (standard) from RIBOBIO served as the siRNA control. The HepG2 cells were transfected with AURKA-siRNA or Ctrl-siRNA using Lipofectamine® RNAiMAX Reagent

(Thermo Fisher Scientific) according to the manufacturer's protocol. After 48 h, the cells were collected and the knockdown effect was evaluated using Western blot.

2.4 Cell Viability Assay

Cell viability was measured by Cell Counting Kit-8 (CCK-8) assay in accordance with manufacturer's instruction. Cells were seeded into 96-well plates at a density of 5000 cells/well, and treated with different concentration of Danusertib. After cultured for 48 h, and 20 μ L CCK-8 solution was added into each well to detect cell viability. The absorbance at 450 nm was measured by a microplate reader.

2.5 Clone Formation Assay

Cells were seeded into 6-well plates at a density of 500 cells per well in RPMI-1640 serum-free medium. The growth medium of each well was carefully changed every 2 days for two weeks, then cells were washed with PBS and stained with crystal violet. Clones of each well were photographed and scored.

2.6 Cell Migration and Invasion Assay

Cells in the logarithmic phase were seeded onto the upper chamber of a Transwell or on a Matrigel-coated Transwell in serum-free medium. The lower chamber contained DMEM with 10% FBS. After 48 h, non-migrated cells were gently removed from the upper chamber by cotton swabs, while migrated cells were stained using 0.1% crystal violet and counted under a microscope.

2.7 Cell Cycle Analysis

Cells were reseeded in 6-well plates at a density of 4×10^5 cells/well). After administration, the cells washed with PBS and incubated with RNase A solution (100 μ L) for 30 min at 37 °C. Then, 400 μ L PI was added and incubated for 30 min at room temperature. The DNA content was detected by flow cytometry (Guava easyCyte HT; Millipore, USA).

2.8 Cell Apoptosis Assay

Cells in the logarithmic phase were washed with PBS and resuspended at 1×10^6 cells/ml, Then the Apoptosis of the cells was examined with eBioscience™ Annexin V Apoptosis Detection Kit (cat. no. 88-8007-74; eBioscience) according to the manufacturer's instructions and analyzed by flow cytometry (Guava easyCyte HT; Millipore, USA).

2.9 qRT-PCR

Total RNA was isolated by TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and cDNA was synthesized using the TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix (cat. no. AT311-02; Transgen Biotech, China) in accordance with the manufacturer's protocol. All cDNAs were amplified by using UltraSYBR Mixture (cat. no. CW2602; CWBIO, China). The PCR condition was as following: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec, 60 °C for 40 sec. Primer sequences were as follows: AUEKA: forward, 5'-CTGCATTTTCAGGACCTGTTAAGG-3' and reverse, 5'-AACGCGCTG GGAAGAATTT-3'; HSF1: forward, 5'-GACCAAGCTGTGGACCCTC-3' and reverse, 5'-

CACTTTCCGGAAGCCATACAT-3'; GAPDH: forward, 5'-TGTGGGCATCAATGGATTTGG-3' and reverse, 5'-ACACCATGTATTCCGGGTCAAT-3'.

2.10 Western Blot

Proteins from the indicated cells were extracted using RIPA lysis buffer (cat. no. P0013B; Beyotime, China) and protein concentrations were determined by the BCA Protein Assay Kit (cat. no. P0010; Beyotime, China). Then proteins were loaded and separated on SDS-PAGE, followed by a transfer onto PVDF membranes (Millipore, Bedford, MA). Membranes were incubated overnight at 4 °C with primary antibodies against AURKA (1:1000; cat. no. 91590; Cell Signaling Technology, Inc.), HSF1 (1:1000; cat. no. 4356; Cell Signaling Technology, Inc.) CDC2 (1:1000; cat. no. 9116; Cell Signaling Technology, Inc.), cyclin B1 (1:1000; cat. no. 12231; Cell Signaling Technology, Inc.), Cleaved caspase3 (1:1000; cat. no. 9664; Cell Signaling Technology, Inc.), Cleaved PARP (1:1000; cat. no. 5625; Cell Signaling Technology, Inc.), ATF4 (1:1000; cat. no. 11815; Cell Signaling Technology, Inc.), p-EIF2 α (1:1000; cat. no. 3398; Cell Signaling Technology, Inc.), EIF2 α (1:1000; cat. no. 5324; Cell Signaling Technology, Inc.) or CHOP (1:1000; cat. no. 2895; Cell Signaling Technology, Inc.), GAPDH (1:1000; cat. no. 5174; Cell Signaling Technology, Inc.) served as loading control. After washed with TBST three times, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:1000; cat. no. A0201; A0208 Beyotime, China) for 1 h at room temperature. Bands densities were measured by the ECL detection system (Tanon, Shanghai, China), then scanned and analyzed with Image J (National Institutes of Health, Bethesda, MD, USA).

2.11 Animal Xenografts Study

Female BALB/c nude mice (6-week-old) were obtained from Hangzhou Ziyuan Experimental Animal Technology co. LTD, and housed in well-ventilated rooms under specific pathogen-free conditions. The animal experiments were approved by the ethics committee of Jinling Hospital (approval no: 2020JLHGKJDWLS-140).

HCC model was constructed by subcutaneously injecting HepG2 cells into the right flank of the mice. After 12 d, the mice were randomly divided into 4 groups and treated with KRIBB11, Danusertib or KRIBB11 combined with Danusertib, respectively. The body weight and tumor volume of mice were recorded every two days. A month after inoculation, all mice were sacrificed and the xenografts were weighted. The tissues of heart, kidney and liver were harvested. Xenograft volume was calculated with using this formula $(V) = 0.5 \times l \times w^2$, in which w is the short diameter, and l is the long diameter. Then the tissues and xenografts were embedded in paraffin and subjected to hematoxylin-eosin (HE) staining and immunohistochemical staining, respectively.

2.12 HE staining

The paraffin-embedded sections were dewaxed, rehydrated, and stained with hematoxylin. After rinsed with running water, the sections were stained with eosin, dehydrated by gradient ethanol, transparent with xylene, and sealed with neutral gum. Then the histopathology of the tumor was observed by light microscope.

2.13 Immunohistochemistry (IHC)

The paraffin-embedded sections were dewaxed with dimethyl benzene and rehydrated in dH₂O. The slides were immersed in citrate buffer (0.01 M pH 6.0), with high pressure heating for 2 min. Then, the slides were incubated with antibody against AURKA (cat. no. 91590; Cell Signaling Technology, Inc.), HSF1 (cat. no. 4356; Cell Signaling Technology, Inc.), Cleaved caspase 3 (cat. no. 9664; Cell Signaling Technology, Inc.), Ki-67 (cat. no. 9449; Cell Signaling Technology, Inc.) or ATF4 (cat. no. 11815; Cell Signaling Technology, Inc.) overnight at 4°C, followed by conjugation to the secondary antibody and DAB staining (cat. no. ZLI-9018; Beijing Zhongshan Golden Bridge Biotechnology Co, Ltd, Beijing, China). Finally, the slides were then counterstained using hematoxylin, dehydrated, and sealed with neutral gum. The staining results were observed by microscope (Nikon, Japan).

2.14 Statistical Analysis

The data were presented as the mean \pm SD and analyzed by SPSS 20.0 software. Group comparisons were performed using Student's t-test or one-way ANOVA with Tukey post-test. The correlation analysis was carried out by Pearson correlation analysis. $P < 0.05$ was considered statistically significant.

3 Results

3.1 High expression of AURKA is associated with poor prognosis of HCC

To investigate the correlation between AURKA expression and the HCC progression, totally 3 pairs of HCC tissue samples and adjacent normal tissues were tested for AURKA expression by qRT-PCR, Western blot, and IHC. The results showed that the expression of AURKA in HCC tissues was significantly higher than that in adjacent normal tissues (Fig. 1A-C). In addition, through online Gepia database analysis (gepia.cancer-pku.cn/detail.php?) of AURKA expression in HCC and adjacent normal tissues, the results revealed that AURKA was highly expressed in HCC (Fig. 1D), and the overall survival of HCC patients with high AURKA expression was significantly lower than that of HCC patients with low AURKA expression (Fig. 1E), which indicated that high AURKA expression was related to the poor prognosis of HCC patients.

3.2 AURKA promotes the proliferation and migration of HCC cells

To validate whether AURKA had pro-oncogenic potentiality for HCC, AURKA knockdown HepG2 cells were successfully constructed (Fig. 2A), and the clone formation ability and migration were detected. As shown in Fig. 2B, Knockdown of AURKA significantly decreased clone formation ability in HepG2 cells. Additionally, knockdown of AURKA significantly inhibited the migration and invasion of HepG2 cells (Fig. 2C-D). Thus, these results suggested that AURKA played an important role on the proliferation and migration of HCC cells.

3.3 AURKA inhibitor (Danusertib) inhibits the proliferation and migration of HCC cells, and promotes the cell apoptosis

Then, the HepG2 cells were treated with different concentrations of Danusertib to explore the effect of AURKA inhibitor (Danusertib) on cellular biology of HCC cells. The results showed that Danusertib effectively inhibited proliferation of HepG2 cells, and it was concentration-dependent (Figure 3A). Besides, compared with control groups, the clone formation, migration and invasion in Danusertib groups were significantly decreased (Figure 3B-D), which was consistent with the effect of AURKA knockdown in HepG2 cells. Then, flow cytometry was used to determine the effects of Danusertib on the apoptosis of HepG2 cells. The results revealed that the HepG2 cells treated with Danusertib had increased apoptosis and the cell cycle was arrested in G2/M phase (Figure 4A, C). Additionally, the protein expression of CDC2 and cyclin B1 was significantly decreased while the expression of Cleaved PARP and Cleaved caspase3 was obviously increased (Figure 4B, D). Therefore, it was concluded that inhibition of AURKA could attenuate the proliferation and induce the apoptosis of HCC cells.

3.4 High HSF1 expression is associated with poor prognosis of HCC and positively correlated with AURKA expression

Furthermore, high expression of HSF1 was observed in the HCC tissues (Figure 5A-D), and the overall survival of HCC patients with high HSF1 expression was significantly lower than that of HCC patients with low HSF1 expression (Figure 1E). Through correlation analysis, it was found that HSF1 expression were significantly positively correlated with AURKA expression in HCC. Therefore, we suspected that simultaneous inhibition of HSF1 and AURKA might significantly suppress HCC development.

3.5 HSF1 inhibitor (KRIBB11) combined with AURKA inhibitor (Danusertib) induces HCC apoptosis by activating endoplasmic reticulum stress

To explore the cancer-promoting mechanisms of HSF1 and AURKA, HepG2 cells were treated with KRIBB11, Danusertib, and KRIBB11+ Danusertib, respectively, and Western blot was used to detect the expression of endoplasmic reticulum stress-related proteins. As shown in Figure 6A, the protein expression of ATF4 and the phosphorylation of EIF2 α in HepG2 cells treated with KRIBB11 or Danusertib were increased, while after treated by KRIBB11 combined with Danusertib, the expression of ATF4, CHOP and p-EIF2 α was notably increased, indicating that endoplasmic reticulum stress of HCC was activated after combined inhibition of AURKA and HSF1. Then, ATF4 knockdown HepG2 cells were constructed and treated with or without KRIBB11+ Danusertib. The results pointed out that knockdown of ATF4 in HepG2 cells reversed the increase in ATF4 expression and apoptosis caused by co-administration (Figure 6B-C). Besides, TUDCA, an inhibitor of endoplasmic reticulum stress, also reversed the increase in ATF4 expression and apoptosis caused by co-administration (Figure 6D-E), which suggested that combined inhibition of AURKA and HSF1 could induce HCC apoptosis by activating endoplasmic reticulum stress.

3.6 HSF1 inhibitor (KRIBB11) combined with AURKA inhibitor (Danusertib) attenuates tumorigenesis and tumor growth of HCC cells *in vivo*

Then, the pro-oncogenesis of AURKA and HSF1 *in vivo* was analyzed by co-administration of KRIBB11 and Danusertib in HCC nude mice. The results revealed that there was no significant difference in body weight of nude mice in four groups (Figure 7A), while the xenograft volume of four groups grew in a time-dependent manner, and the xenografts in KRIBB11+Danusertib group grew significantly slower than that in other three groups (Figure 7B). At the ending point, xenografts were collected and weighted. The xenografts in Ctrl group were obviously big and heavy, while the xenografts in KRIBB11+Danusertib group was significantly smaller and lighter (Figure 7C). Through HE staining, it was found that there was no significant difference in the pathological morphology of heart, kidney and liver in the four groups, indicating that KRIBB11 and Danusertib had lower drug toxicity (Figure 7D). Besides, the results of immunohistochemistry showed that compared with Ctrl group, the Ki67 protein expression in KRIBB11+Danusertib group was reduced and the expression of Cleaved caspase3 and ATF4 were obviously increased (Figure 7E). The above experiments proved that combined inhibition of AURKA and HSF1 could attenuate the tumorigenesis of HCC cells.

4 Discussion

AURKA is a cell cycle regulating kinase, which plays key role in regulating many links of mitosis, and the activity of AURKA significantly increases during the transition from G2 to M phase[4]. Studies have reported that AURKA is closely related to the occurrence and development of endometrial cancer, colorectal adenocarcinoma, oral cancer, and other malignant tumors[19–21]. In this study, we also confirmed that AURKA was highly expressed in HCC tissues and cells, and significantly related to the poor prognosis of HCC. In recent years, the important role of AURKA in cell cycle regulation and the use of small molecule inhibitors make it expected to become an effective target for anti-cancer therapy[22].

Following the discovery of the first AURKA inhibitor ZM447439 as a potential drug for cancer targeted therapy, more than 30 AURKA inhibitors have been introduced into cancer therapy[22]. Here, we evaluated the anti-tumor effect of the AURKA inhibitor Danusertib on HCC, and the results showed the after Danusertib treatment, the proliferation, migration and invasion of HCC cells were significantly inhibited in a dose-dependent manner. Besides, Danusertib induced G2/M cell cycle arrest by activating the Cdc2/cyclin B1 pathway and cell apoptosis by promoting PARP and caspase-3 cleavage. Therefore, the AURKA inhibitor Danusertib could exhibit significant anti-tumor effects on HCC, which indicated that AURKA might become a potential target for HCC treatment.

HSF1 is also a potential therapeutic target for liver cancer, which can induce tumorigenesis and development by promoting cell proliferation, inhibiting cell apoptosis and suppressing anti-tumor immunity[23]. In liver cancer, HSF1 is involved in regulating the cell cycle, growth, and colony formation of liver cancer cells. The results in this study found that HSF1 was also highly expressed in HCC tissues, and there was significant correlation between HSF1 and AURKA expression, so we studied the effect of simultaneous inhibition of AURKA and HSF1 on HCC. The *in vivo* study results revealed that compared with control group or HCC model treated with Danusertib or KRIBB11, combined inhibition of AURKA and HSF1 notably suppressed the HCC tumor growth in HCC nude mice model with relative low drug-associated cytotoxicity, suggesting that combined inhibition of AURKA and HSF1 could have a better HCC efficacy.

In order to further explore the potential mechanism of jointly inhibiting AURKA and HSF1 to induce HCC cell apoptosis, we focused on the endoplasmic reticulum stress (ERS) of HCC cells treated with AURKA and HSF1 inhibitors. The occurrence of HCC is usually closely associated with chronic inflammation and chronic liver damage related to liver disease. In the process of abnormal differentiation of liver cells, these pathogenic factors can induce ERS through oxidative stress, inflammation, and gene mutations[24]. Early ERS can alleviate the cell damage caused by stress by activating the unfolded protein response (UPR). Studies have shown that HCC can adapt to various unfavorable environments by activating UPR, thereby conducive to tumor cell survival [24]. However, when continuously in the ERS state, ERS will trigger the apoptosis program, causing cancer cell apoptosis. Lin et al.[25] found that HCC cells HepG2 had a stress response and increased apoptosis under the induction of tunicamycin, which further explained the role of ERS in inducing HCC cell apoptosis. Jin et al.[26] found that after mice were fed with hexavalent chromium, the expression of GRP78, ATF6 and CHOP in liver tissues increased, which promoted cell apoptosis, and there was a certain correlation with the dosage. According to reports, the CHOP pathway of ER stress played an important role in the apoptosis of HCC cells induced by kaempferol[27]. In this study, we demonstrated that co-inhibition of AURKA and HSF1 led to increased expression of ERS-related proteins in HCC cells, such as ATF4, p-eIF2 α and CHOP. Theoretically, phosphorylation of eIF2 α leads to selective translation of ATF4. Besides, in the case of prolonged ERS state, the induction of several pro-apoptotic genes mediated by ATF4-CHOP will further induce cell apoptosis[28]. Therefore, we suggested that co-inhibition of AURKA and HSF1 can induce HCC apoptosis by activating endoplasmic reticulum stress, thereby inhibiting tumor progression.

5 Conclusions

Combined inhibition of AURKA and HSF1 suppresses HCC development through reducing apoptosis by activating endoplasmic reticulum stress, which provides a new target for HCC therapy.

List Of Abbreviations

AURKA

aurora kinase A; HSF1:heat shock transcription factor 1; HCC:hepatocellular carcinoma; IHC:immunohistochemistry; ERS:endoplasmic reticulum stress; FBS:fetal bovine serum; siRNA:small-interfering RNA; CCK-8:Cell Counting Kit-8; HE:hematoxylin-eosin; UPR:unfolded protein response.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Jinling Hospital, and the written informed consent were obtained from patients. The animal experiments were approved by the Ethics Committee of Jinling Hospital (approval no: 2020JLHGKJDWLS-140).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

ZS and XH conceived and designed this study; LY, HZ, XJ and CJ contributed to the experiment and analysis of the data; ZS and XZ drafted the manuscript; XH provided critical comments, suggestions and revised the manuscript. All authors read and approved the final version of the manuscript.

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Figures

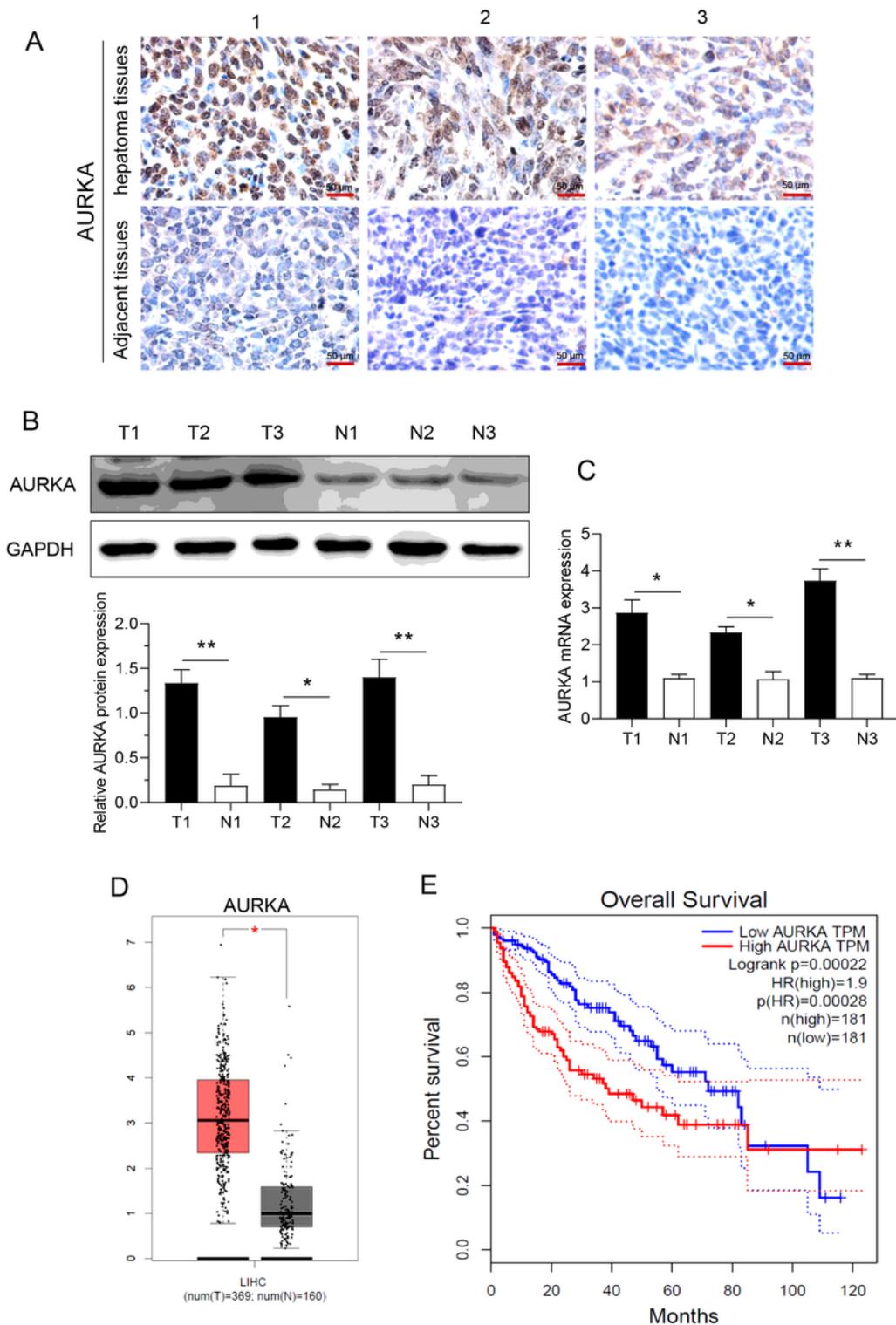


Figure 1

High expression of AURKA is associated with poor prognosis of HCC (A) Immunohistochemical staining for AURKA in HCC and adjacent normal tissues. (B) qRT-PCR and (C) Western blot were performed to determine the AURKA expression in the HCC and adjacent normal tissues, GAPDH served as internal control. (D) AURKA expression in HCC and adjacent normal tissues obtained from online database. (E)

Kaplan-Meier curves of the overall survival of HCC patients with low or high AURKA expression. *P<0.05; **P<0.01.

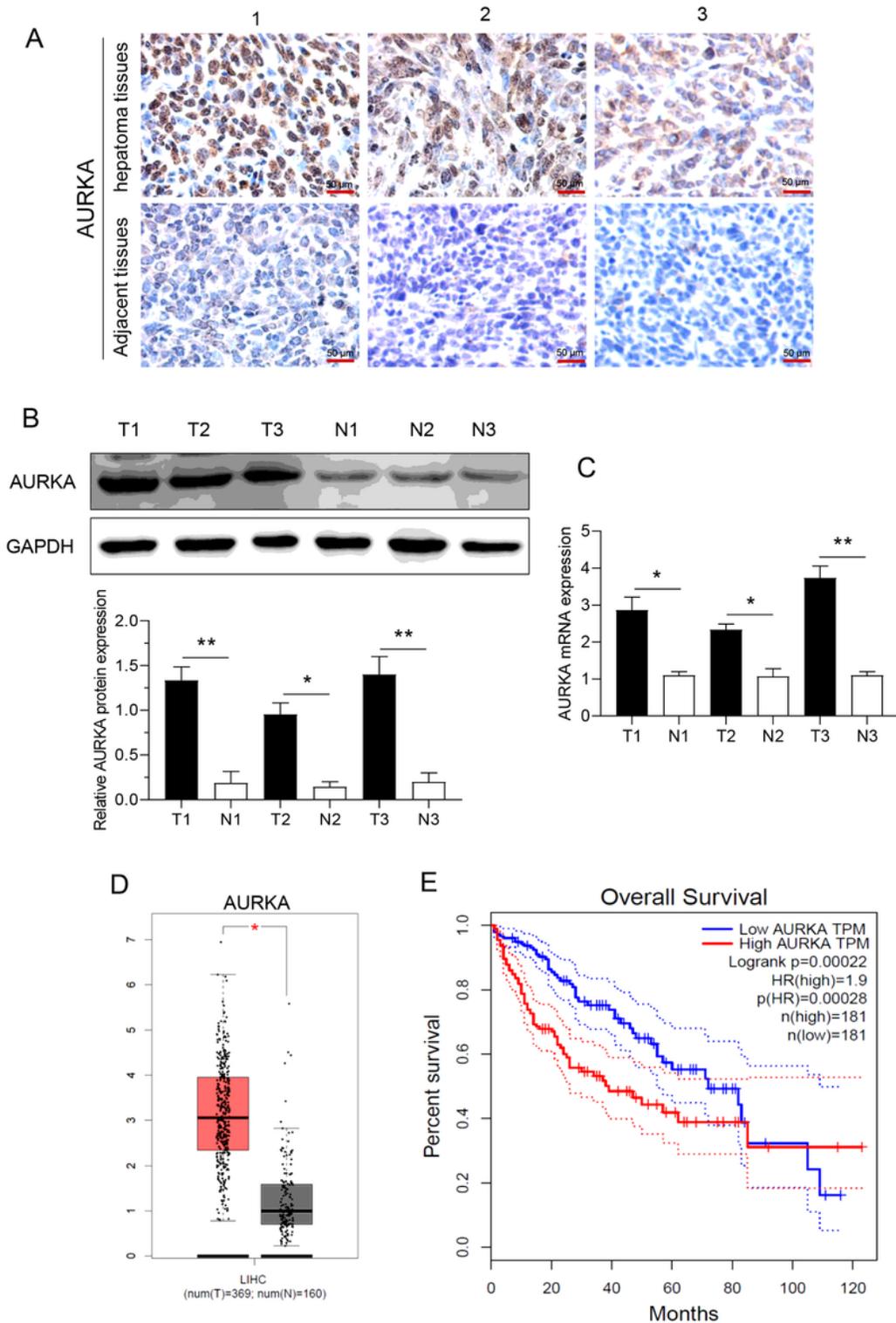


Figure 1

High expression of AURKA is associated with poor prognosis of HCC (A) Immunohistochemical staining for AURKA in HCC and adjacent normal tissues. (B) qRT-PCR and (C) Western blot were performed to determine the AURKA expression in the HCC and adjacent normal tissues, GAPDH served as internal

control. (D) AURKA expression in HCC and adjacent normal tissues obtained from online database. (E) Kaplan-Meier curves of the overall survival of HCC patients with low or high AURKA expression. * $P < 0.05$; ** $P < 0.01$.

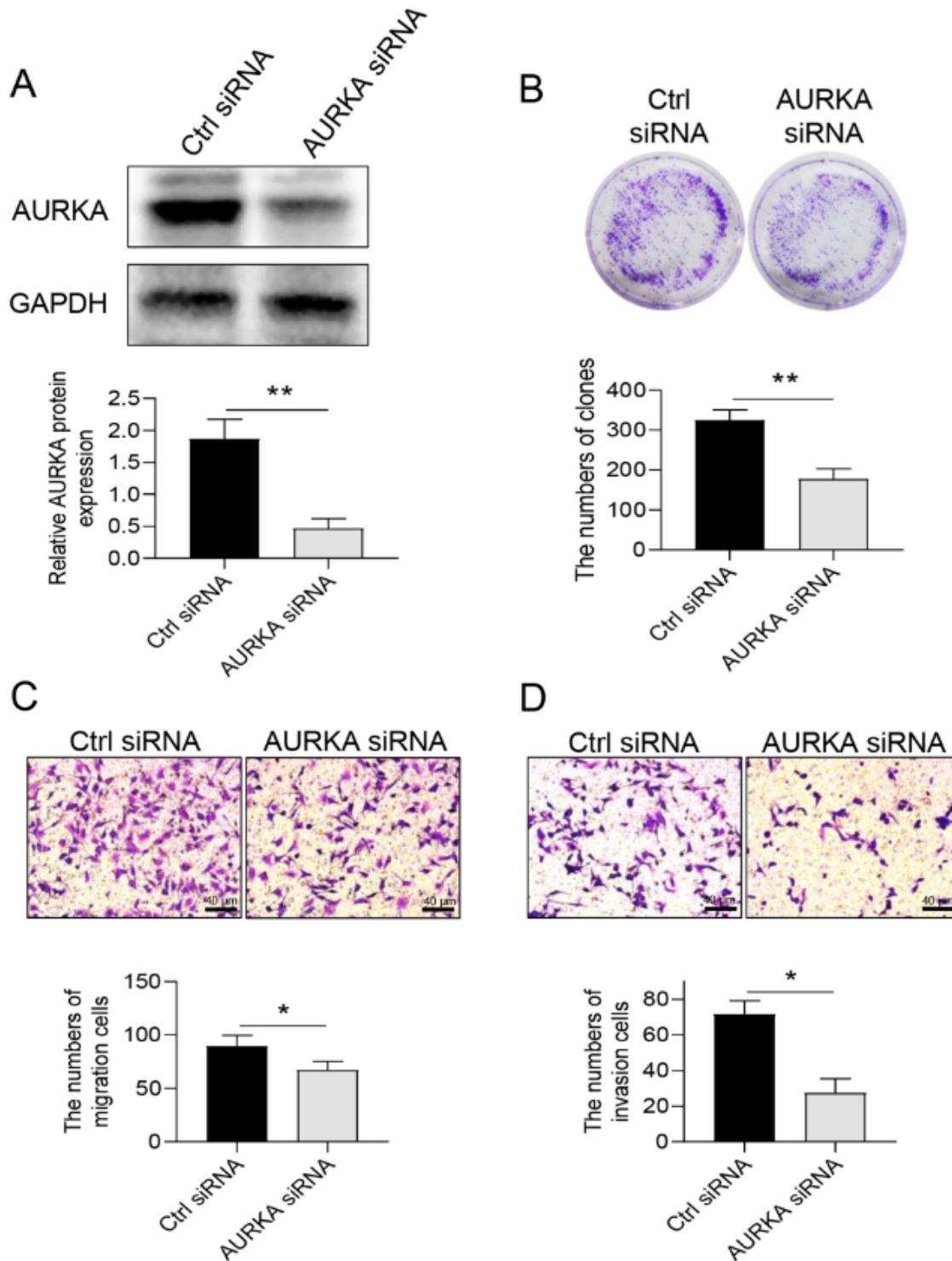


Figure 2

AURKA promotes the proliferation and migration of HCC cells (A) Western blot was used to examine the expression of AURKA in HepG2 cells treated with siRNA targeting AURKA or a negative control, GAPDH

served as internal control. (B) Representative images of colony formation in HepG2 cells treated with siRNA targeting AURKA or a negative control. (C-D) Transwell assays were used to detect the (C) migration and (D) invasion of HepG2 cells treated with siRNA targeting AURKA or a negative control. *P<0.05; ** P<0.01.

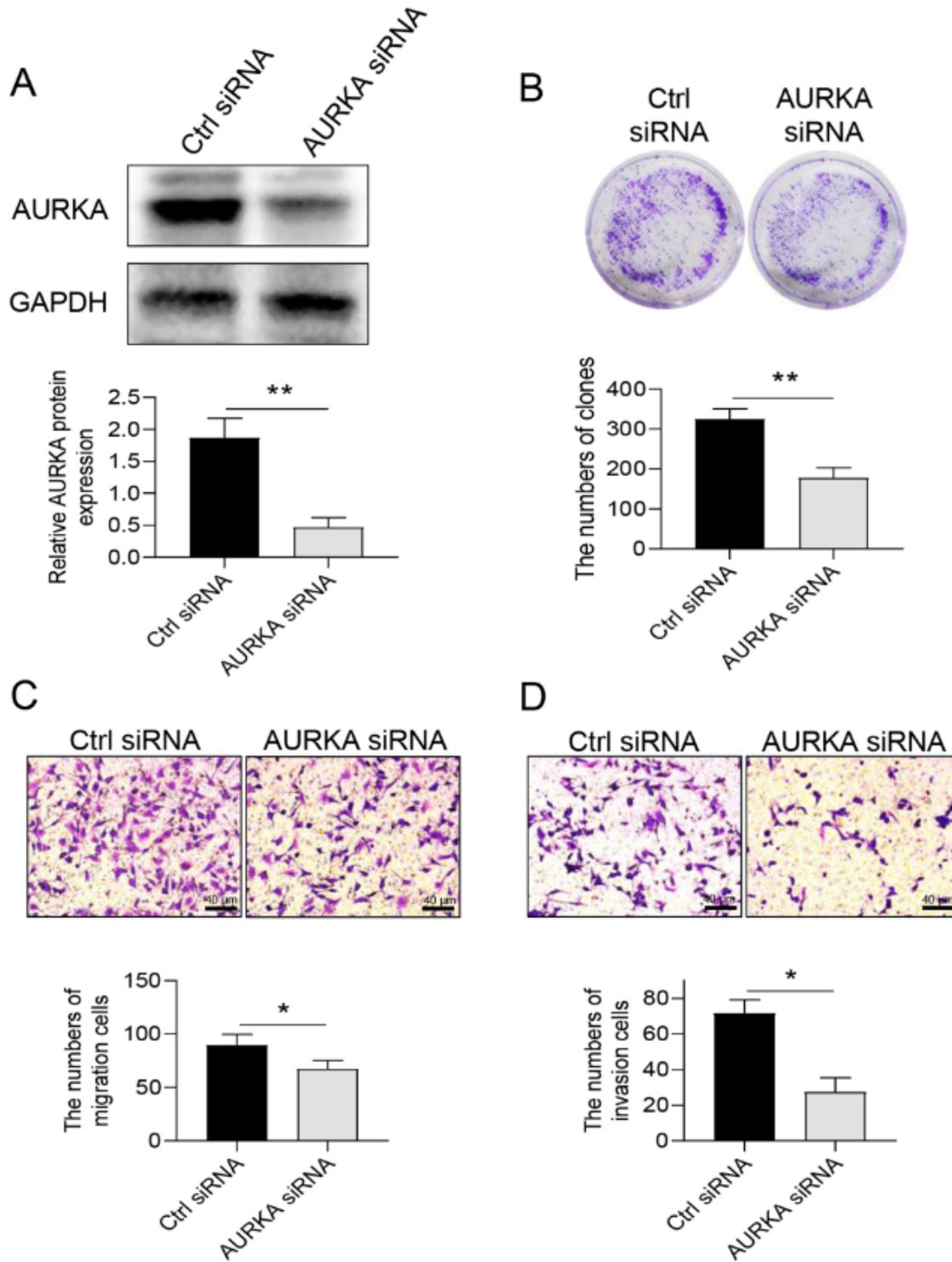


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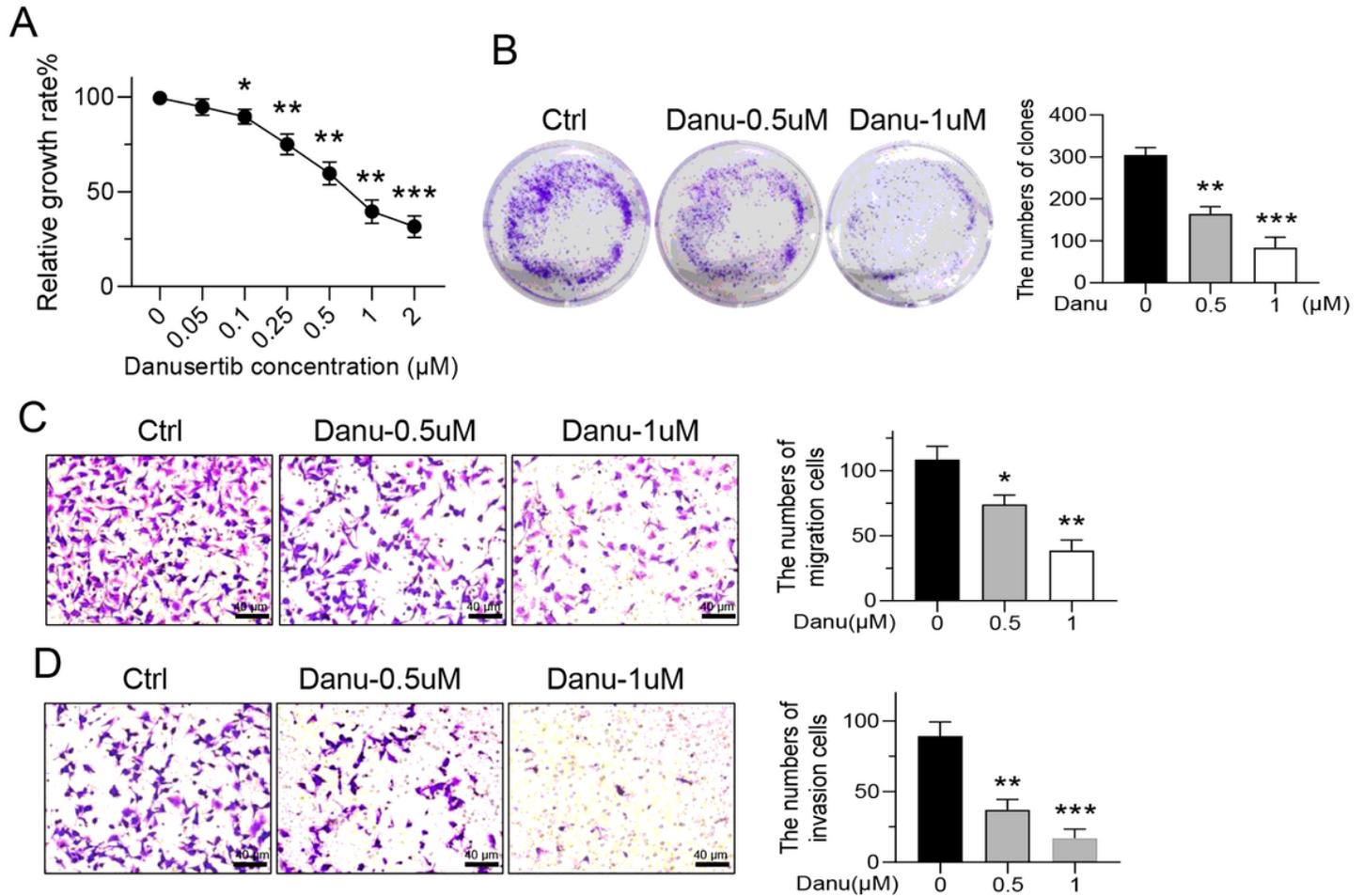


Figure 3

AURKA inhibitor (Danusertib) inhibits the proliferation and migration of HCC cells (A) CCK-8 assay was applied to determine the proliferation of HepG2 cells treated with different concentrations of Danusertib (0, 0.05, 0.1, 0.25, 0.5, 1 and 2 μM) for 48 h. (B) Representative images of colony formation in HepG2 cells treated with different concentrations of Danusertib (0, 0.5 and 1 μM) for 48 h. (C-D) Transwell assays were used to detect the migration and invasion of HepG2 cells treated with different concentrations of Danusertib (0, 0.5 and 1 μM) for 48 h. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

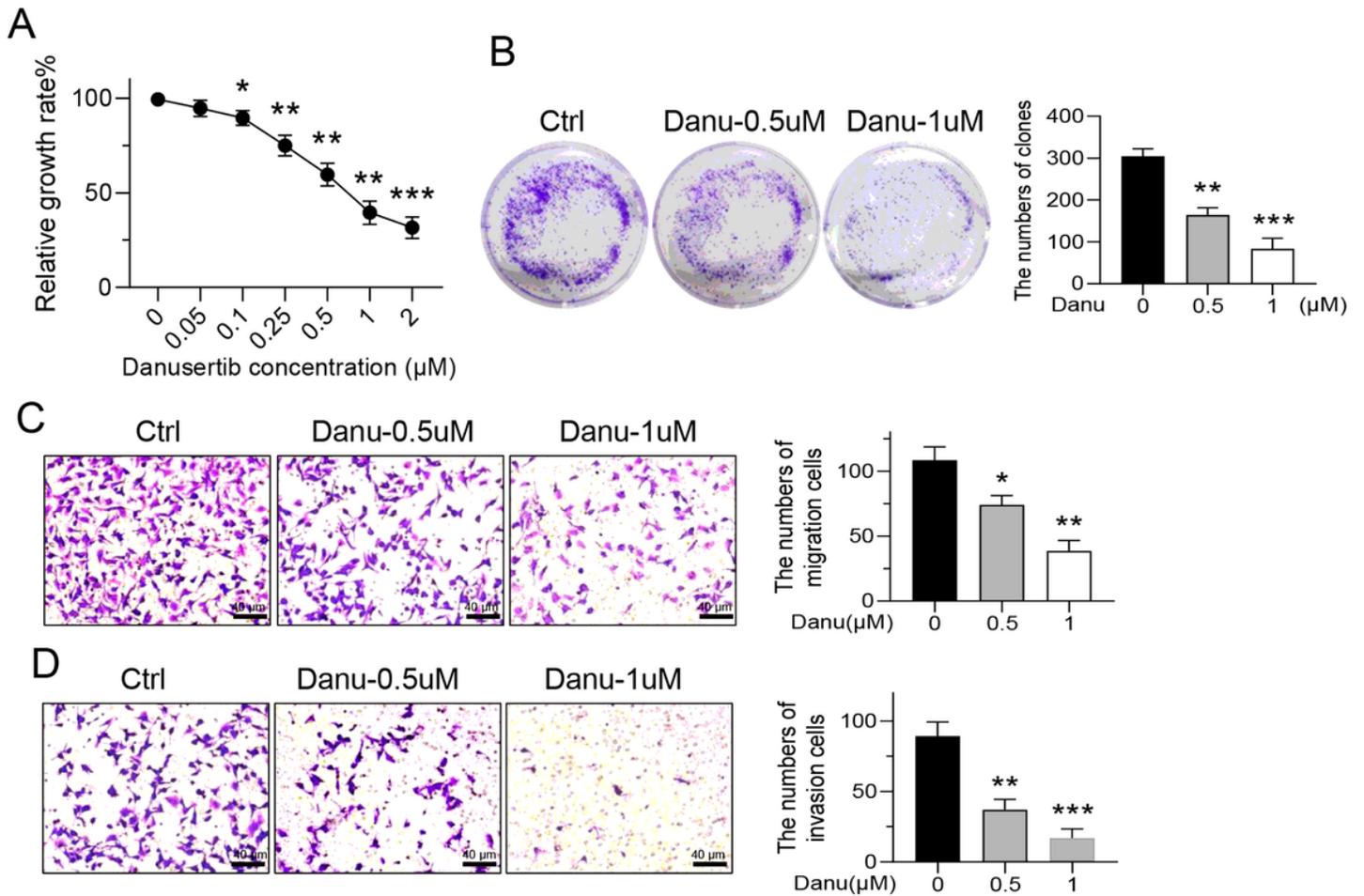


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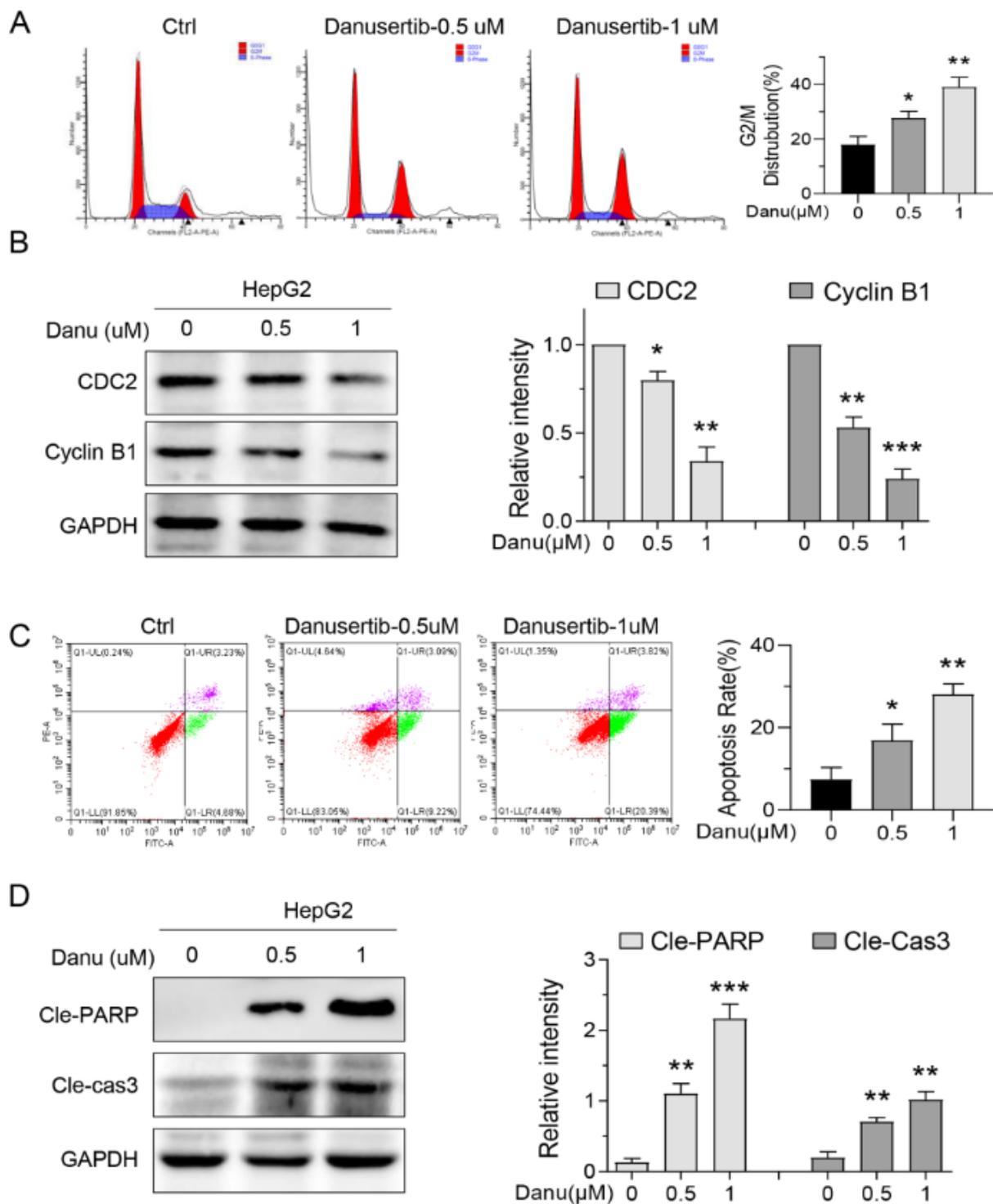


Figure 4

AURKA inhibitor (Danusertib) induces apoptosis of HCC cells (A) Flow cytometric detection of cell cycle in HepG2 cells treated with different concentrations of Danusertib (0, 0.5 and 1 μ M) for 20 h. (B) Western blot was used to detect the expression of CDC2 and Cyclin B1 in HepG2 cells treated with different concentrations of Danusertib (0, 0.5 and 1 μ M) for 20 h. GAPDH served as internal control. (C) Flow cytometric detection of apoptosis in HepG2 cells treated with different concentrations of Danusertib (0,

0.5 and 1 μ M) for 24 h. (D) Western blot was used to examine the expression of Cleaved PARP and Cleaved caspase3 in HepG2 cells treated with different concentrations of Danusertib (0, 0.5 and 1 μ M) for 24 h, GAPDH served as internal control. * P <0.05; ** P <0.01; *** P <0.001.

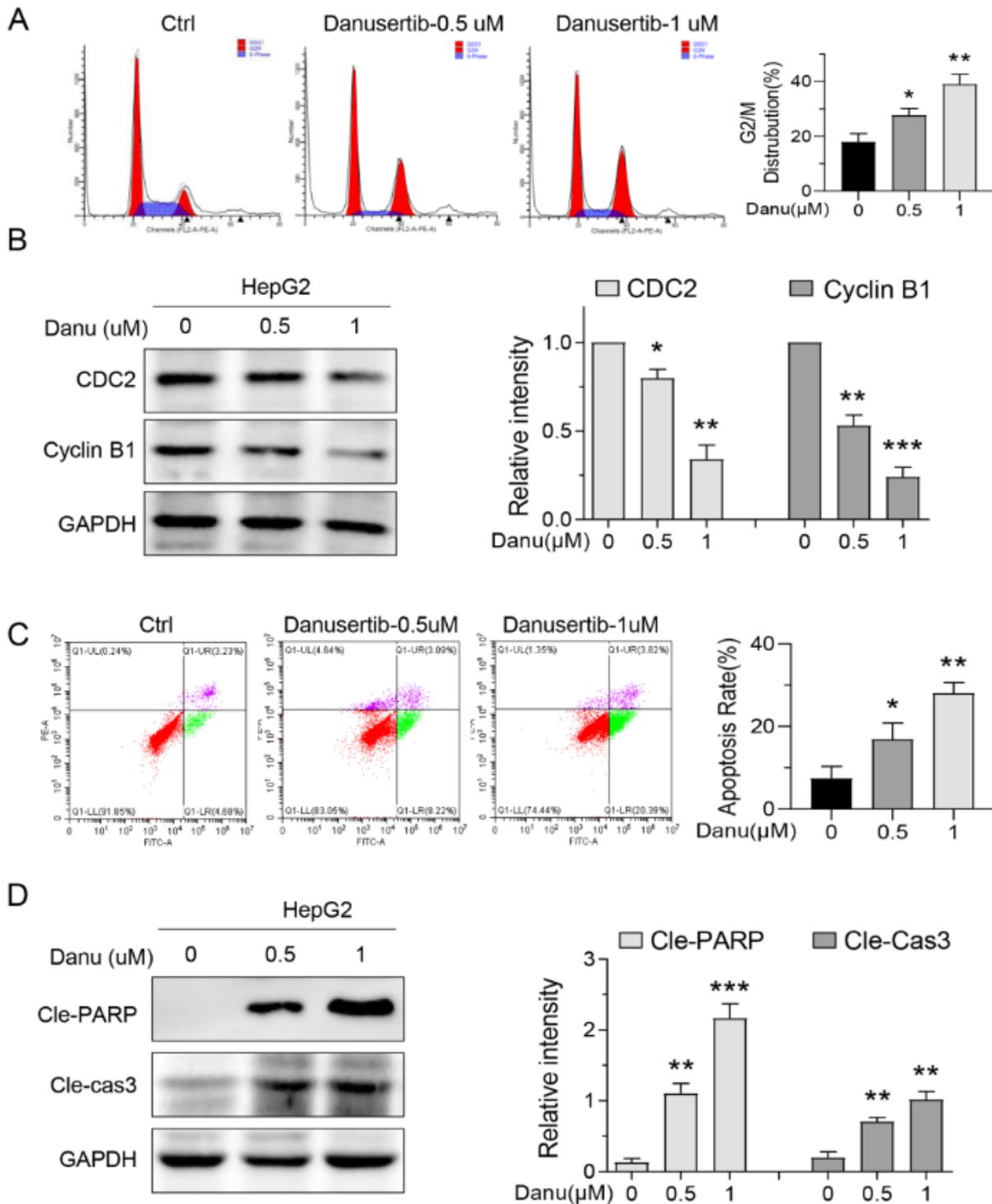


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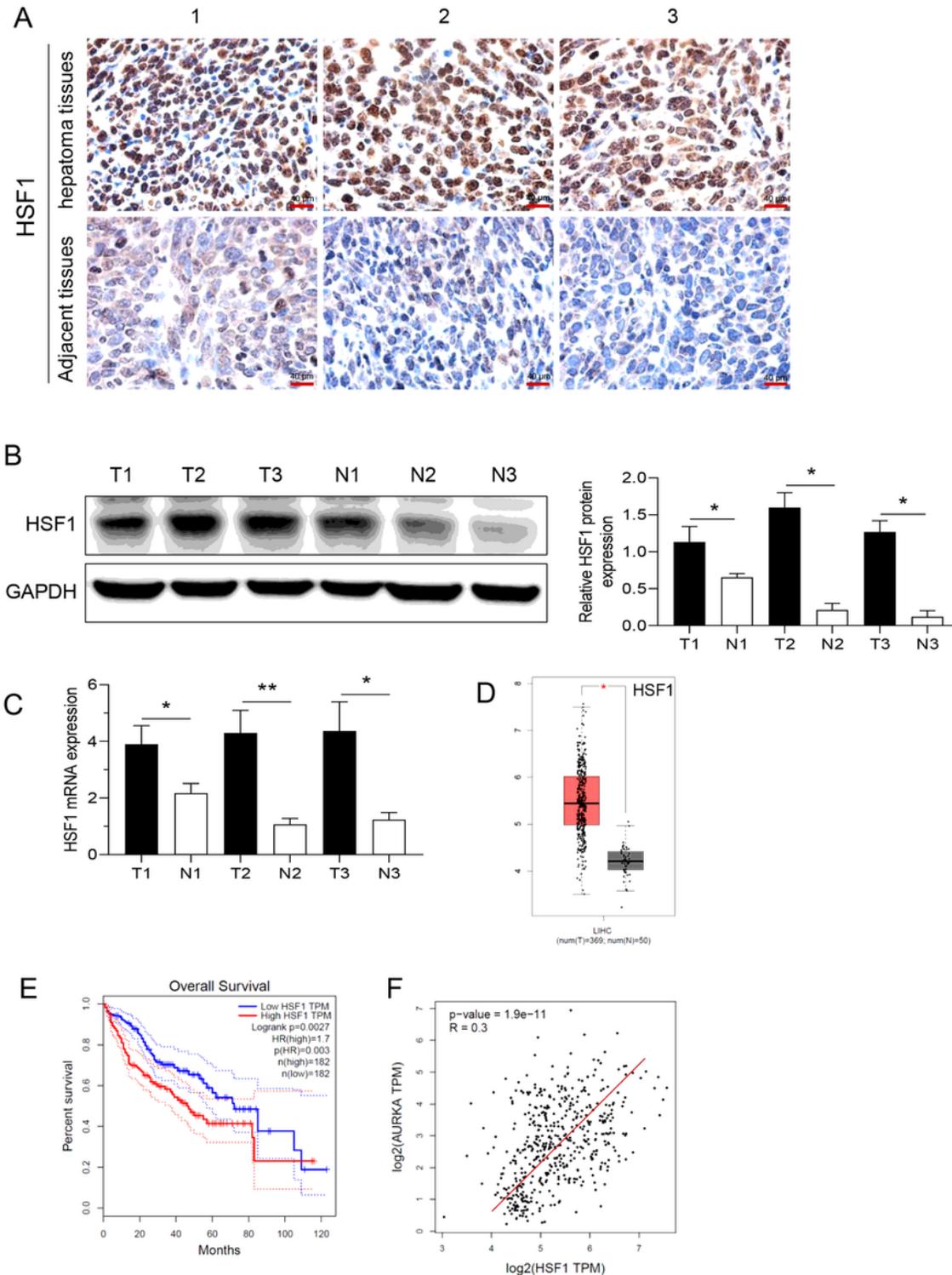


Figure 5

High HSF1 expression is associated with poor prognosis of HCC and positively correlated with AURKA expression (A) Immunohistochemical staining for HSF1 in HCC and adjacent normal tissues. (B) RT-PCR and (C) Western blot were performed to determine the HSF1 expression in the HCC and adjacent normal tissues, GAPDH served as internal control. (D) HSF1 expression in HCC and adjacent normal tissues obtained from online database. (E) Kaplan-Meier curves of the overall survival of HCC patients with low or high HSF1 expression. (F) The correlation between HSF1 and AURKA. * $P < 0.05$; ** $P < 0.01$.

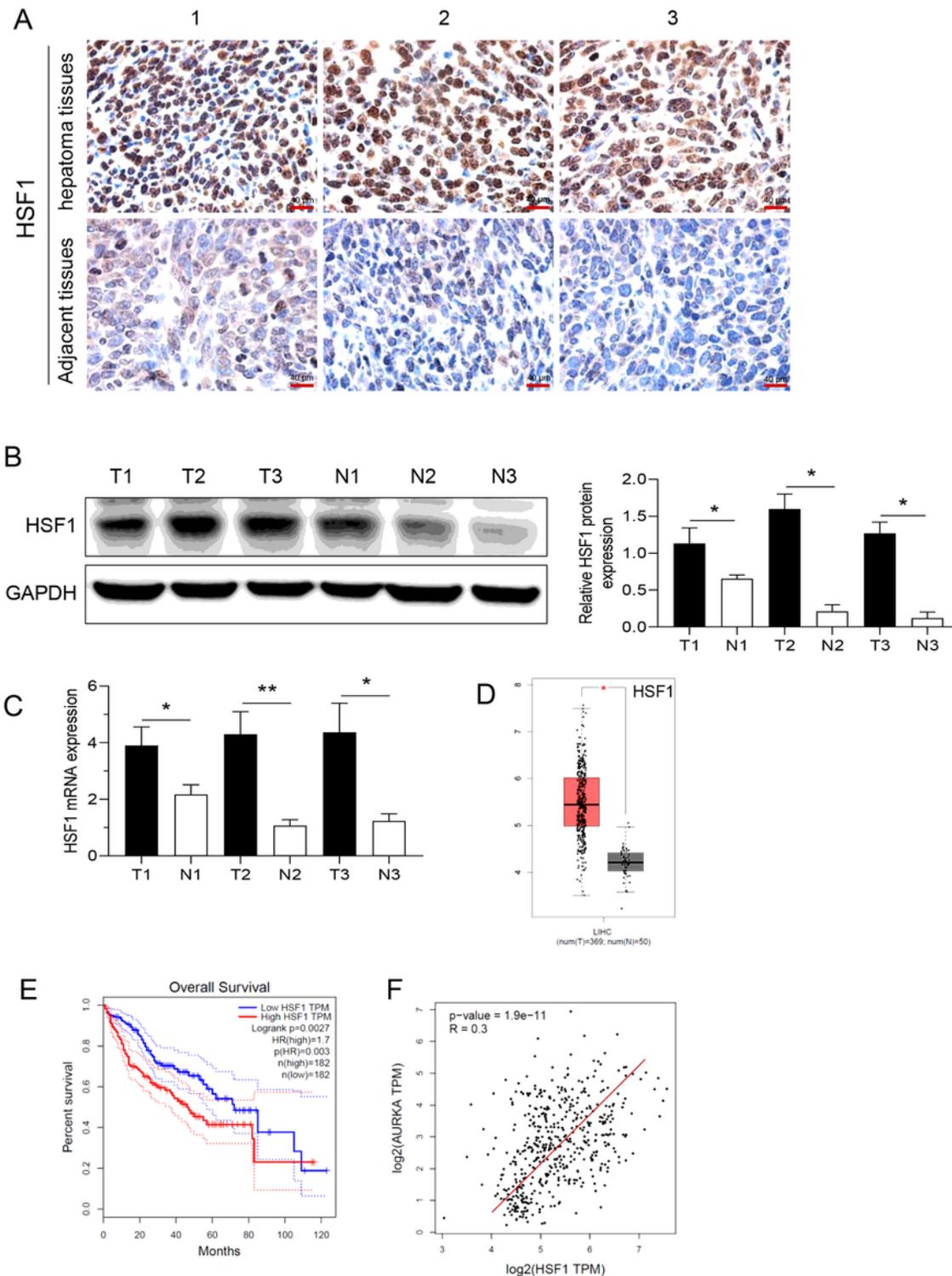


Figure 5

High HSF1 expression is associated with poor prognosis of HCC and positively correlated with AURKA expression (A) Immunohistochemical staining for HSF1 in HCC and adjacent normal tissues. (B) RT-PCR and (C) Western blot were performed to determine the HSF1 expression in the HCC and adjacent normal tissues, GAPDH served as internal control. (D) HSF1 expression in HCC and adjacent normal tissues obtained from online database. (E) Kaplan-Meier curves of the overall survival of HCC patients with low or high HSF1 expression. (F) The correlation between HSF1 and AURKA. * $P < 0.05$; ** $P < 0.01$.

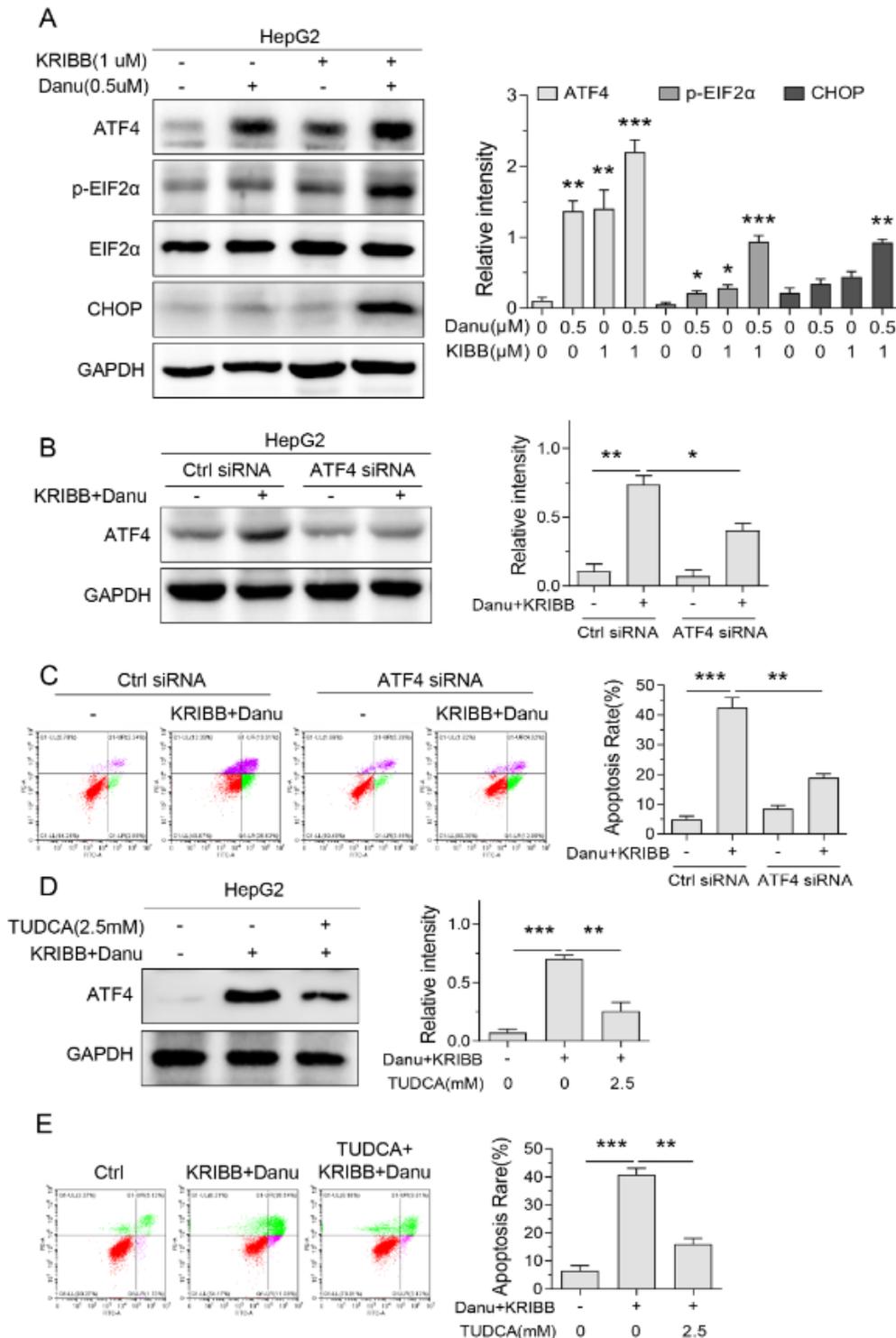


Figure 6

HSF1 inhibitor (KRIBB11) combined with AURKA inhibitor (Danusertib) induces HCC apoptosis by activating endoplasmic reticulum stress (A) Western blot was used to detect the expression of endoplasmic reticulum stress-related protein in HepG2 cells treated with different administration, GAPDH served as internal control. (B) Western blot was performed to determine ATF4 expression in HepG2 and ATF4 knockdown HepG2 cells treated with or without co-administration of KRIBB11 and Danusertib, GAPDH served as internal control. (C) Flow cytometry was used to determine apoptosis of HepG2 and ATF4 knockdown HepG2 cells treated with or without co-administration of KRIBB11 and Danusertib. (D) Western blot was used to determine ATF4 expression in HepG2 cells treated with different administration, GAPDH served as internal control. (E) Flow cytometry was performed to examine apoptosis of HepG2 cells treated with different administration. *P<0.05; **P<0.01; ***P<0.001.

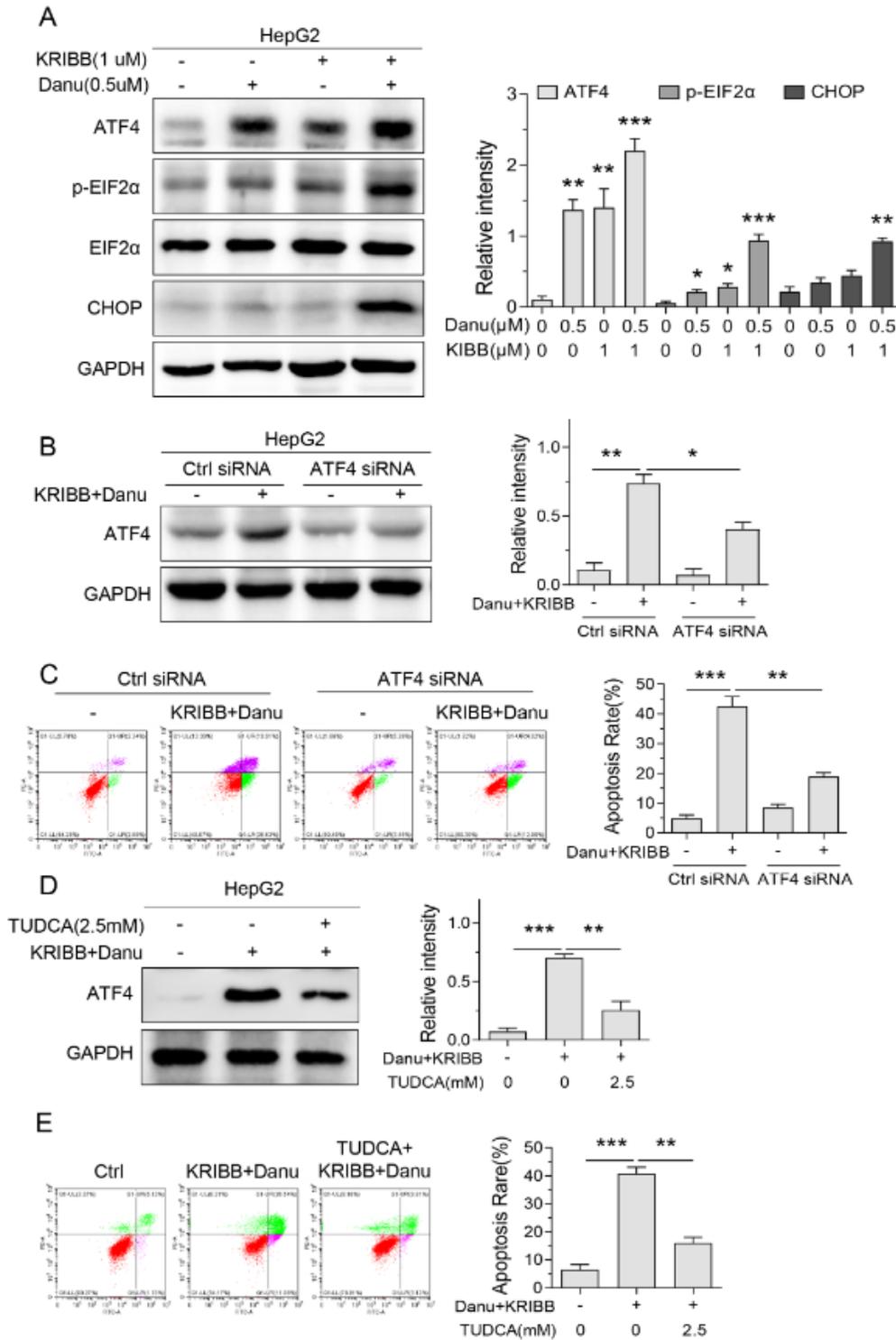


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GAPDH served as internal control. (C) Flow cytometry was used to determine apoptosis of HepG2 and ATF4 knockdown HepG2 cells treated with or without co-administration of KRIBB11 and Danusertib. (D) Western blot was used to determine ATF4 expression in HepG2 cells treated with different administration, GAPDH served as internal control. (E) Flow cytometry was performed to examine apoptosis of HepG2 cells treated with different administration. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

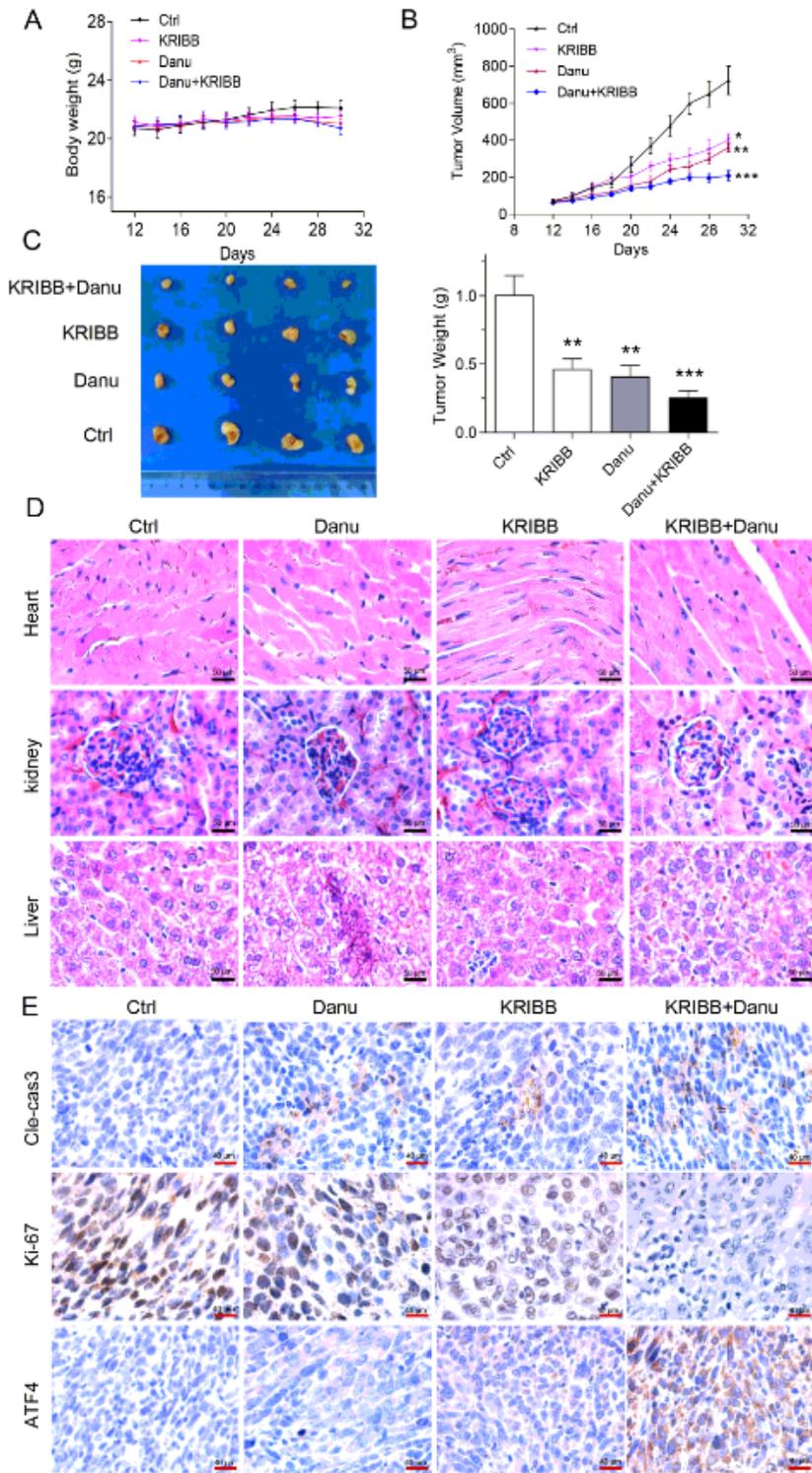


Figure 7

HSF1 inhibitor (KRIBB11) combined with AURKA inhibitor (Danusertib) attenuates tumorigenesis and tumor growth of HCC cells in vivo (A) The body weight curves of mice injected with HepG2 cells. (B) The tumor growth curves of xenografts from HepG2 cells. (C) Xenografts from HepG2 cells were present at the ending point and weighted. (D) HE staining of heart, kidney and liver obtain from nude mice treated with KRIBB11, Danusertib, and KRIBB11+ Danusertib, respectively. (E) Immunohistochemical staining for Cleaved caspase3, Ki-67 and ATF4 in HCC tissues obtain from nude mice treated with KRIBB11, Danusertib, and KRIBB11+ Danusertib, respectively. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

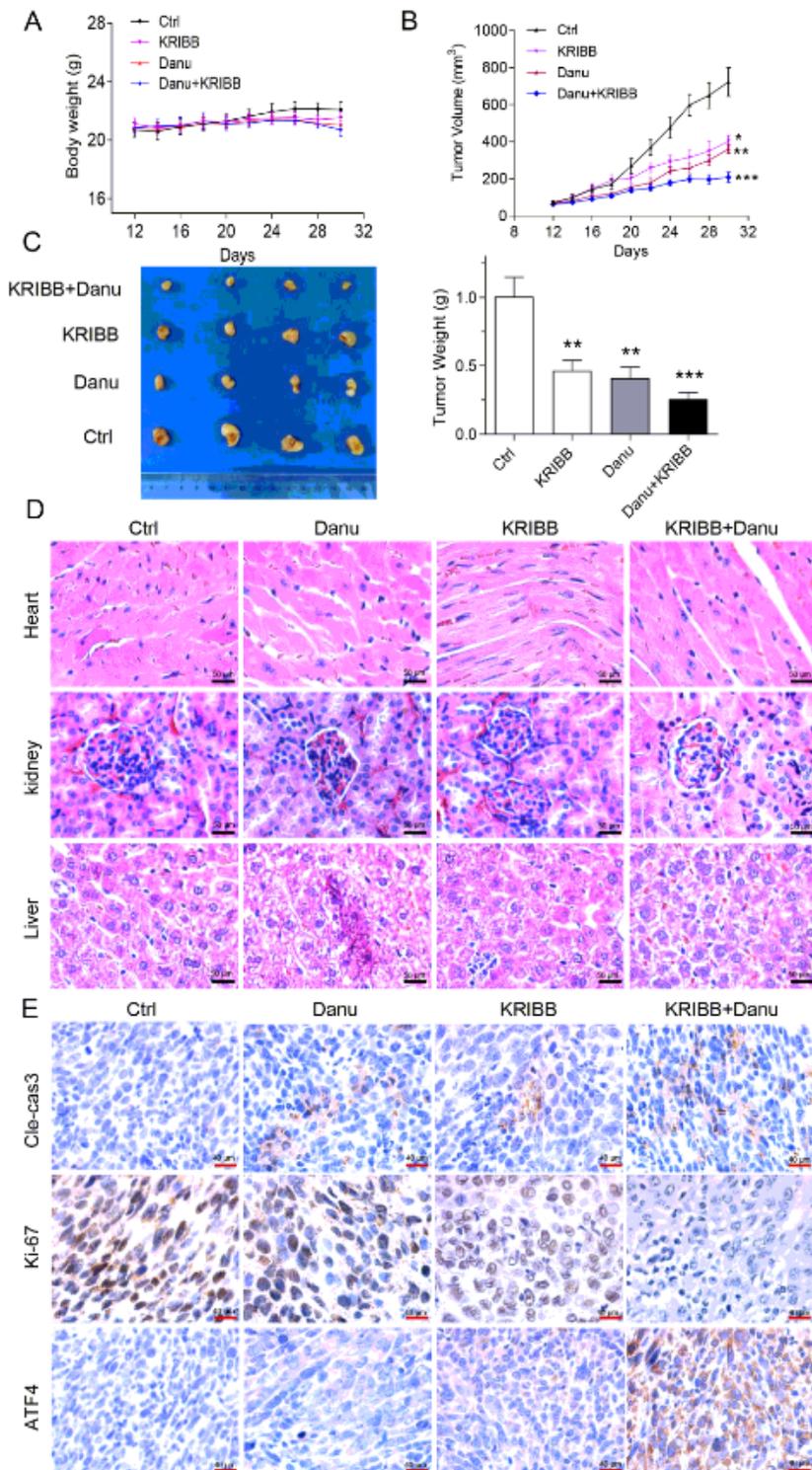


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