Local hyperthermia inhibits proliferation of hepatocellular carcinoma cells via regulating the Hippo signaling pathway

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Abstract

Hepatocellular carcinoma (HCC) is the third malignant tumor in the world. Many evidences have demonstrated that hyperthermia exerts a crucial role in cancer therapy. However, it remains elusive whether hyperthermia restrains HCC progression and the underlying molecular mechanism of hyperthermia on HCC is unknown. Here, we find that local hyperthermia has a strong anti-liver tumor effect *in vivo* and *in vitro*. Heat stimulus decreases the proliferation of liver cancer cells *in vitro* and suppresses the tumor growth in mice through inducing apoptosis. Furthermore, RNA-seq results have demonstrated that the Hippo signaling pathway plays a major role in heat therapy. Mechanistically, heat stimulation restrains the expression of YAP protein in the nucleus. Together, all our results have suggested that heating therapy inhibits the proliferation of HCC cells by regulating the Hippo signaling pathway, suggesting YAP is a potentially promising target for HCC therapy.

Introduction

Hepatocellular carcinoma(HCC)is one of the most common malignant types of liver cancer and the third common cause of cancer-related death in the world[1]. Only 5% -15% patients can be cut off the liver in hepatectomy due to the high degree of malignancy[1, 2].In addition, another major reason is that the tumor cells can spread from a primary site to other distant sites of patients, resulting in tumors formation in different organs or tissues. To date, radiotherapy, chemotherapy, immunotherapy and targeted therapy are still the most popular methods used by patients. However, the overall 5-year survival rate remains less than 20%[3]. Thus, it is urgent and necessary for us to develop new strategies or targets for HCC therapy.

Hyperthermia is a common treatment method in clinical, high-temperature can kill the tumor cells without affecting normal cells through artificial heating[4]. Now it has been believed that hyperthermia exerts its anti-tumor mechanisms or therapeutic efficacy by different mechanisms such as the destruction of tumor cell membrane, the change of tumor cytoskeleton, DNA, RNA and protein synthesis inhibition, apoptosis induction and tumor angiogenesis inhibition and enhanced immunity[5, 6]. According to the different scopes of action, hyperthermia can be divided into three different types including local hyperthermia, regional hyperthermia and whole-body hyperthermia[7]. Local hyperthermia is often used to treat solid tumors especially for Squamous Cell Carcinoma, such as head and neck tumors[8]. But regional hyperthermia is suitable for relatively large areas, for example, recurrent ovarian cancer can be treated by intraperitoneal thermochemotherapy and metastatic esophageal squamous cell carcinoma can be treated by regional thermoradiotherapy[9, 10]. Moreover, systemic hyperthermia is usually used in combination with other methods in advanced metastatic malignancies to strengthen therapy efficacy[11]. In recent years, thermotherapy has been applied to different types of cancer therapy including breast cancer, esophageal cancer, bladder cancer, lung cancer and head and neck cancer, and so on[12]. It has been reported that high temperature exposure with 42℃ inhibits the proliferation of colorectal cancer cell HCT116through inducing apoptosis by increasing the expression of miR-34a and p53[13]. In addition, Zhao et al have demonstrated that hyperthermia with 43 ℃ inhibits the proliferation of non-small cell

lung carcinoma (NSCLC) H460, PC-9 and H1975 cells through promoting caspase-3-dependent apoptosis mediated by ATM-Chk2 cdc25Cpathway. Wu et al also found that hyperthermia treatment with 42 ℃ significantly inhibits the proliferation of A549 cells by promoting the expression of p21 and Bax mRNA et al[13–15]. All these results indicate that hyperthermia is a promising strategy for cancer therapy. However, all findings also have demonstrated that different cancer needs to use different temperature, treatment time and dose in clinical[16]. Thus, it is important to investigate the antitumor role of hyperthermia in different cancer types and explore the underlying mechanisms.

The Hippo signaling pathway was firstly found in Drosophila melanogaster and played a crucial role in controlling cell growth, proliferation, apoptosis, differentiation, and organ size[17, 18]. In recent years, many studies have shown that abnormal activation of Hippo signaling pathway results in the development of a variety of tumors. For example, the over-activation and amplification of YAP/TAZ have been observed in lung cancer, colorectal cancer, pancreatic cancer, breast cancer and glioma[19]. It was reported that ZNF123 negatively regulated the progression of triple negative breast cancer through Hippo/YAP signaling by promoting the ubiquitination of YAP K48; Zhang et al found that Hippo/YAP could promote the proliferation and colony formation of OSCC cells by activating FRA-1 and metastases of lung and pancreatic cancer by miR-19b-3p[20–22]. Moreover, the Hippo signaling pathway is a key regulator of liver metabolism and homeostasis. Meanwhile, the dysregulation of YAP / TAZ is closely related to liver development, regeneration, and tumorigenesis[23]. The results of clinical samples indicated that Yap expression was higher in liver cancer than that in normal tissues, and YAP was an independent risk factor for the prognosis of HCC. Inhibiting the expression of YAP reduces the proliferation and migration of liver cancer cells[24–26]. Chen et al found that Alpha Actinin1, which competitively interacted with MOB1, promoted the growth of HCC through reducing the phosphorylation of LATS1 and YAP[27]. In addition, microRNA-375 inhibited the proliferation and invasion of HCC by directly decreasing the expression of YAP protein[27, 28]. Moreover, growing evidence has indicated that the Hippo pathway plays an important role in regulating the occurrence and development of HCC by multiple signaling pathways cooperation including interacting with Wnt pathway, Notch pathway and TGF-β pathway[29, 30]. However, whether hyperthermia could be a potent therapeutic strategy against HCC and the underlying mechanisms are still largely unknown.

Here our results indicate that heating therapy has a strong anti-liver tumor effect in vivo and in vitro. Heat stimulus decreases the proliferation of liver cancer cells in vitro and suppresses the tumor growth in mice through inducing apoptosis. Furthermore, RNA-seq results have demonstrated that the Hippo signaling pathway plays a major role in heat therapy. Mechanistically, the results from cytoplasmic separation and immunofluorescence show that the expression of YAP protein in the nucleus was reduced after heat stimulation. Together, all our results have suggested that heating therapy inhibits the proliferation of HCC cells by regulating the Hippo signaling pathway and YAP is a potentially promising target for HCC therapy.

Materials And Methods

Cell lines and heating

The HCC cell lines Huh7 and HepG2 were obtained from the American Type Culture Collection (Manassas, VA, USA). All the cells were cultured in DMEM (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc.) at 37°C with 5% CO_2. The experiment was grouped according to the temperature. Set 40 ℃, 43 ℃ 46 ℃ experimental group and 37 ℃ control group. The cells in the logarithmic growth period which were grown in the culture bottle were sealed with parafilm and heated in a constant temperature incubator at a set temperature for 1 hour. Then, the cells were incubated at 37 ℃ with 5% CO_2.

Cell counting kit-8 (CCK-8) assay

Cells following hyperthermia were collected and plated on 96-well plates. 24 h, 48 h, 72 h, 96 h or 120h later, 10μl CCK-8 reagent was added to each well and then the plate was incubated for 2 h at 37°C. After that, the absorbance was measured at 450 nm using a Vmax microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). Each sample was assayed in triplicate and repeated 3 times independently.

Colony Formation Assay

The indicated cells were trypsinized and seeded on 6-well plates (500 cells/well) and cultured for 2 weeks. The colonies were stained with Hematoxylin for 30 min after fixation with 4% paraformaldehyde for 30 min. The number of colonies, defined as > 50 cells/colony, was counted. Three independent experiments were performed.

Flow cytometry

$1x10^6$ indicated cells were collected and fixed with 70% cold ethanol. After centrifugation, the cells were washed once and incubated with 0.5 ml of propidium iodide (PI) staining buffer containing 200 mg/ml RNaseA and 50 μg/ml PI (Beyotime Biotechnology Co., Ltd, China) at 37 °C for 30 min in the dark. Cell cycle distribution was analyzed by FACScan cytometry (Becton-Dickinson, San Jose, CA, USA).

And a flow cytometer was used to assess cell apoptosis with an Annexin-V-FITC Apoptosis Detection kit (Beyotime Biotechnology Co., Ltd, China). After heat treatment, the cells were harvested and washed twice with cold PBS prior to 10^5 cells being resuspended in 200 μl binding buffer supplemented with 10μl Annexin-V FITC and 5μl PI. The cells were then incubated in the dark for 10 min and a flow cytometric analysis was performed.

ICG (Indocyanine green) and its cytotoxicity

The photothermal performance for each of the aqueous solutions of the formulation with different ICG concentrations（0, 50,100, 200, 400 and 600 ug/ml）individually was measured after 808 nm laser irradiation (1W/cm2) for a period of 600s. And its cytotoxicity was detected by using CCK8.

Animal experiments

4 to 6-week-old BABL/c female nude mice were purchased from the Center of Laboratory Animal Science of Beijing Weitong Lihua (Beijing, China). All animal experiments were conducted according to the National Institutes of Health (NIH) Guidelines for Laboratory Animal Care and approved by the Xinxiang Medical University Institutional Animal Care and Use Committee. Xenograft tumors were generated by subcutaneous injection of HCC cells. When the tumor size reached about 80 mm^3, the nude mice were randomly divided into 3 groups, Saline only (NC group); Saline and exposed to the near-infrared laser (NIR group); ICG and exposed to the near-infrared laser (ICG+NIR). The tumor volumes were measured by a caliper every 3 days for 21 days and were calculated by the formula: 1/2 × (length × $width^2$). On day 21, major organs, together with tumors were removed, fixed in 4% formalin and then processed for Immunohistochemical staining studies for pathological features.

RNA-seq

Total RNA of HepG2 treatment with 37℃ and 43℃ cells was extracted using RNAiso Plus reagent (Takara, Kusatsu, Shiga, Japan). RNA-seq and bioinformatics were performed in The Beijing Novogene Institute (Novogene, Beijing, China). RNA degradation and contamination were monitored on 1% agarose gels. RNA purity was checked using the NanoPhotometer ® spectrophotometer (IMPLEN, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). A total amount of 1 μg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following the manufacturer's recommendations and index codes were added to attribute sequences to each sample.

Differential expression analysis and KEGG pathway annotation

Differential expression analysis of two conditions/groups (two biological replicates per condition) was performed using the DESeq2 R package (1.16.1). DESeq2 provides statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting P-values were adjusted using Benjamini and Hochberg's approach for controlling the false discovery rate. Genes with an adjusted *p*-value <0.05 found by DESeq2 were assigned as differentially expressed. Then the differential expression genes were analyzed by using KEGG pathway analysis.

Western blotting (WB)

Total proteins were extracted from HCC cells. The cells were lysed in cold lysis buffer (60mM Tris-HCl at pH 7.4, 150mM NaCl, 0.25% SDS and 1% Tergitol-type NP-40) containing 10mM NaF, 1mM Na_3VO_4 and complete protease inhibitor (Roche Diagnostics, Basel, Switzerland) for 30 min on ice, and were then centrifuged at 12,000 × g at 4°C for 15 min as previously described. A bicinchoninic acid protein assay was used to determine protein concentration. The proteins were subjected to SDS-PAGE and transferred

onto a PVDF membrane. The PVDF membrane was subsequently blocked in PBST solution containing 5% non-fat milk and incubated at 4°C overnight with anti-bax, anti-bcl-2, anti Caspase3 P17/P19, anti-P21, anti-P27, anti-Cyclin D1, anti-YAP, (1:1000 dilution; ProteinTech Group, Chicago, IL, USA) and anti-Tubulin (1:1000 dilution; Beyotime, Shanghai, China). The next day, membranes were incubated with the corresponding HRP-conjugated secondary antibody (dilution 1:5000; ABclonal Biotech Co., Ltd., Woburn, MA, USA). Subsequently, the membranes were detected using Pierce ECL Western Blotting Substrate (Thermo Scientific, USA).

Separation of cytoplasm and nucleus fraction

Nuclear and cytosolic RNAs were separated using the PARIS Kit (Beyotime, Shanghai, China) according to the manufacturer's instructions. Details are provided in supplementary material, Supplementary materials and methods.

Immunofluorescence analysis

Cells were seeded onto coverslips at a density of 5×10^4 with primary antibodies against YAP (Proteintech Group, Chicago, MA, USA) overnight at 4°C. The coverslips were then incubated with rhodamine-conjugated or fluorescein isothiocyanate (FITC)-conjugated goat antibodies against rabbit or mouse IgG (Proteintech Group, Chicago, MA, USA). After counterstaining with DAPI (Beyotime, Shanghai, China), images were taken using an Olympus FV1000 confocal laser-scanning microscope (Olympus America Inc, NY, USA). The experiment was performed with three replicates.

Results

Effects of hyperthermia at different temperatures on the inhibition of HCC cell proliferation

To determine the effect of hyperthermia on HCC cells proliferation. CCK-8 assay was performed according to the manufacturer. As shown in Fig.1, The results of CCK8 showed that the proliferation of Huh7 cells cultured at 43℃ or 46℃ for 48h, 72h, 96h or 120h were all inhibited significantly compared with the 37℃ control group (Fig. 1A and B), and the proliferation of HepG2 cells cultured at 43℃ or 46℃ for 24h, 48h, 72h, 96h or 120h were all inhibited significantly compared with the 37℃ control group (Fig. 1C and D). The results of colony formation assay also revealed that the colony numbers of Huh7 and HepG2 cells cultured at 43℃ were significantly reduced compared with the 37℃ control group (Fig. 1E and F).

Hyperthermia promotes apoptosis and inhibits the cell cycle of HCC cells

One of the early changes of apoptosis is the transfer of phosphatidyl serine (PS) from the inside of the cell membrane to the outside. Annexin-V is a Ca^+ dependent phospholipid binding protein with a high affinity for PS[31]. However, the transfer of PS from inside of the cell membrane to the outside is not unique to apoptosis, it can also occur in cell necrosis. Apoptotic cells can resist all the dyes used for cell

activity identification such as PI, while the necrotic cells can't. Therefore, the normal cells are double negative for Annexin-V and PI, the apoptotic cells are Annexin-V positive and PI negative, and the necrotic cells are double positive for Annexin-V and PI. Liver cancer cells were treated with thermal stimulation (43 ℃, 48h), and the proportion of cells in normal survival, early apoptosis and late apoptosis was analyzed by flow cytometry. The results showed that compared with the control group, the total number of apoptosis of Huh7 cells increased from 4.0% to 26.8% (Fig. 2A and B). The results were also the same in HepG2 cells, the total apoptosis of HepG2 cells increased from 2.5% to 24.6% (Fig. 2C and D). Subsequently, the distribution of HCC cells within the stages of the cell cycle was examined by flow cytometry. Cells cultured at 43℃ showed a decrease in the percentage of cells in the S peak. The DNA is synthesized in the S phase, and the decrease in the S phase indicates that cell proliferation is inhibited (Fig. 2E-H). The proportion of S-phase cells in Huh7 cells decreased from 32.92% to 26.66% (Fig. 2E and F). The percentage of HepG2 cells in G1 phase increased from 58.30% to 62.17%, and the proportion of S phase cells decreased from 24.94% to 17.91% (Fig. 2G and H). These results suggested that hyperthermia could promote HCC cell proliferation by prohibiting apoptosis and promoting cell cycle progression.

Effects of hyperthermia on the inhibition of HCC cell proliferation in vivo

Different concentrations of ICG were prepared and the heating effects were tested. The results showed that 50 ug / ml ICG had good heating effect, the temperature increased to 43 ℃within 8 minutes, and had no effect on the growth of hepatoma cells (Fig. 3A and B). The CCK8 was used to examine the cytotoxicity of ICG. The results revealed that ICG aqueous solution has low cytotoxicity. Even if the concentration reaches 600ug/ml, more than 80% of the cells still survive (Fig. 3B). Subsequently, we choose 50μg/ml ICG for the in vivo experiment. The nude mice were randomly divided into 3 groups, Saline only (NC group); exposed to the Near-infrared laser (NIR group); ICG and exposed to the Near-infrared the laser (ICG+NIR group). When the xenograft tumor size reached about 80 mm^3, the NIR laser was induced. It revealed an increased effect in thermal efficacy. The temperature of tumors in the ICG+NIR group rises to 43℃ in 5 minutes (Fig. 3C).

The tumor volumes were measured every 3 days for 21 days. It revealed that the volumes and weight of the tumors formed by HCC cells in ICG+NIR group were lower than the NC group (Fig. 4A-D). Immunohistochemistry (IHC) confirmed that tumors of the NC group displayed significantly higher Ki-67 expression than those in the ICG+NIR group (Fig. 4E).

Local hyperthermia could regulate the Hippo signaling pathway

RNA-seq and Bioinformatics analyses were used to explore the underlying mechanisms of local hyperthermia in the apoptosis and proliferation of HCC cells. The results of RNA-seq revealed that 737 genes were up-regulated, while 347 genes were down-regulated in the hyperthermia vs. the control group (Fig. 5A). All the differentially expressed genes (DEGs) with $p < 0.05$ were analyzed by Bioinformatics analyses. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis may be used to determine which signaling pathways are associated with these DEGs. $P < 0.05$ was considered to indicate

a statistically significant difference. It revealed that compared with the control group, the genes of Hippo signaling pathway in 43 ℃ heat treatment group were abundant and significantly up-regulated (Fig. 5B).

The Hippo signaling pathway is involved in regulating apoptosis and cell proliferation. Western blotting was used to evaluate the expression of the key signatures on the Hippo signaling pathway. It illustrated that YAP, CyclinD1 and bcl-2 were significantly downregulated when the HCC cells were cultured at 43℃ compared with the control group. Moreover, bax, Casepase-3P17/P19, P21 and P27 were upregulated when the HCC cells were cultured at 43℃ (Fig. 5C). Subsequently, Western blotting (Fig. 6A and B) and immunofluorescence (Fig. 6C) were used to examine the nucleation of YAP. The results showed the expression of YAP in the nucleus was decreased when the HCC cells were cultured at 43℃ (Fig. 6C). These results suggested that local hyperthermia may promote apoptosis and inhibits the proliferation of HCC cells via regulating the Hippo signaling pathway.

Discussion

Hyperthermia, also named thermal therapy, is a common treatment method that directly or indirectly kills tumor cells by heating alone or in combination with other treatment methods (such as radiotherapy, chemotherapy and targeted therapy)[32–34]. It has been widely used in clinical treatment for different cancers. In this study, we showed that heating treatment suppresses the cell viability and proliferation of HepG2 and Huh7 in a temperature and time dependent manner. Mechanistically, the high temperature was found to inhibit cells proliferation by inducing cell cycle arrest and apoptosis (Fig. 1, 2). More importantly, heating suppressed HepG2 and Huh7 cancer cells survival and proliferation partially through inhibition of YAP (Fig. 5, 6). Taken together, our study uncovers an unappreciated therapeutic action of heating by inhibiting the Hippo pathway in the treatment of HCC.

Cell cycle arrest and apoptosis were reported to be associated with tumor sensitivity to hyperthermia. Several studies suggest that hyperthermia predicts enhanced tumor inhibition through cell growth caused by DNA damage and reduction of synthesis of DNA, RNA, and protein [35, 36]. Wernicki *et al* have reported that high-temperature exposure interrupts the cell growth and promotes apoptosis by induced DNA and RNA damage[37]. Gu *et al* confirmed that heat stress can induce apoptosis through mitochondrial in the p53-dependent pathway [38]. Qi *et al* reported that high temperature with 42 ℃ inhibits the proliferation and promotes apoptosis of renal cell carcinoma (RCC) by inducing G2 / M phase cell cycle arrest[39]. In this study, Our results have suggested that hyperthermia promotes the cell cycle arrest and apoptosis of HepG-2 and Huh7 (Fig. 2). Moreover, in consistent with the findings of Ahmed K[40], high temperature of 43 ℃ does not have any influence on normal cells, but significantly inhibits the growth of HepG2 and Huh7 (Fig. 2). However, some studies have found that hyperthermia with 43 ℃ can't kill cancer cells, such as in human glioma cells T98G, YKG-1 and most gastric cancer cell lines[41, 42]. The possible reason may be lies in the sensitivity of tumor cells to heating. Especially different cell types have different genetic characteristics. More importantly, the methods and materials of tumor hyperthermia also exert an important role in tumor therapy. To date, the common methods of tumor hyperthermia include ultrasound,

microwave, radiofrequency, and magnetic induction [43–46]. However, all these methods have many limitations in clinical therapy. Photothermal therapy (PTT) is an emerging method for tumor treatment due to the advantages of less trauma, fewer adverse reactions, high specificity, and anti-metastasis[47]. It can convert light energy into heat energy under near-infrared light (NIR) irradiation[48]. Given stronger tissue penetration, weak self fluorescence, low interference, low toxicity, high affinity, ICG is approved by the U.S. Food and Drug Administration (FDA) for clinical use in photothermal therapy[49–51]. In this study, indocyanine green (ICG) was used as photothermal conversion material. Our results have demonstrated that ICG has high photothermal conversion efficiency and low cytotoxicity after laser treatment (Fig. 3). Further study found that ICG plus NIR inhibits tumor growth (Fig. 4), which provides the evidence of Photothermal therapy for HCC treatment. Recently, some groups demonstrated that designed nanoparticles with ICG or combination with other immune checkpoint blockade significantly enhance the anti-tumor efficiency of ICG hyperthermia. So, it is worthy to explore whether combination of ICG with other agents also can increase the efficacy of ICG hyperthermia and inhibit metastasis and prevent tumor recurrence in the future[52, 53].

The Hippo signaling pathway is crucial in controlling cell growth and proliferation and so on and YAP/TAZ is a major player in the Hippo pathway. Recent studies have shown that the activity of YAP/TAZ was upregulated in different cancers including liver[19, 23].In addition, YAP is involved in the proliferation, invasion and epithelial mesenchymal transformation (EMT) of tumor cells[54]. Herein, our results from RNA-seq indicated that Hippo signaling pathways were significantly affected or changed after hyperthermia treatment (Fig. 5). Several studies including Luo et al have shown that heat stress induced dephosphorylation and activation of YAP through inducing ubiquitination and degradation of LATS [55]. In line with these findings, our studies also indicated that the expression of YAP decreased after thermal stimulation. In addition, thermal therapy affected the function of the Hippo pathway by reducing the import of the YAP into the nucleus (Fig. 6). However, it is intriguing to test if thermal therapy modulates other signaling pathways in Hippo pathway-independent.

In conclusion, our results have shown that hyperthermia can be used as cancer therapy, at least for HCC. Although our results provide a new promising target of the treatment strategy for liver cancer, further studies are necessary to explore and validate the optimal temperature, time and IGC dose of hyperthermia.

Declarations

Acknowledgments

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Author Contributions

Feng Ren and Yuhan Hu designed the research, Tao Han, Yuhan Hu, Feng Ren and Jian Li wrote the manuscript, Yingying Yang, Yu Zhang, Yuanhua Qin, Jingli Shang and Lifang Wang conducted the experiments and performed the data analysis. All authors discussed the results and reviewed the manuscript.

Declaration of Conflicting Interests

Authors declare no competing financial interests.

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Figures

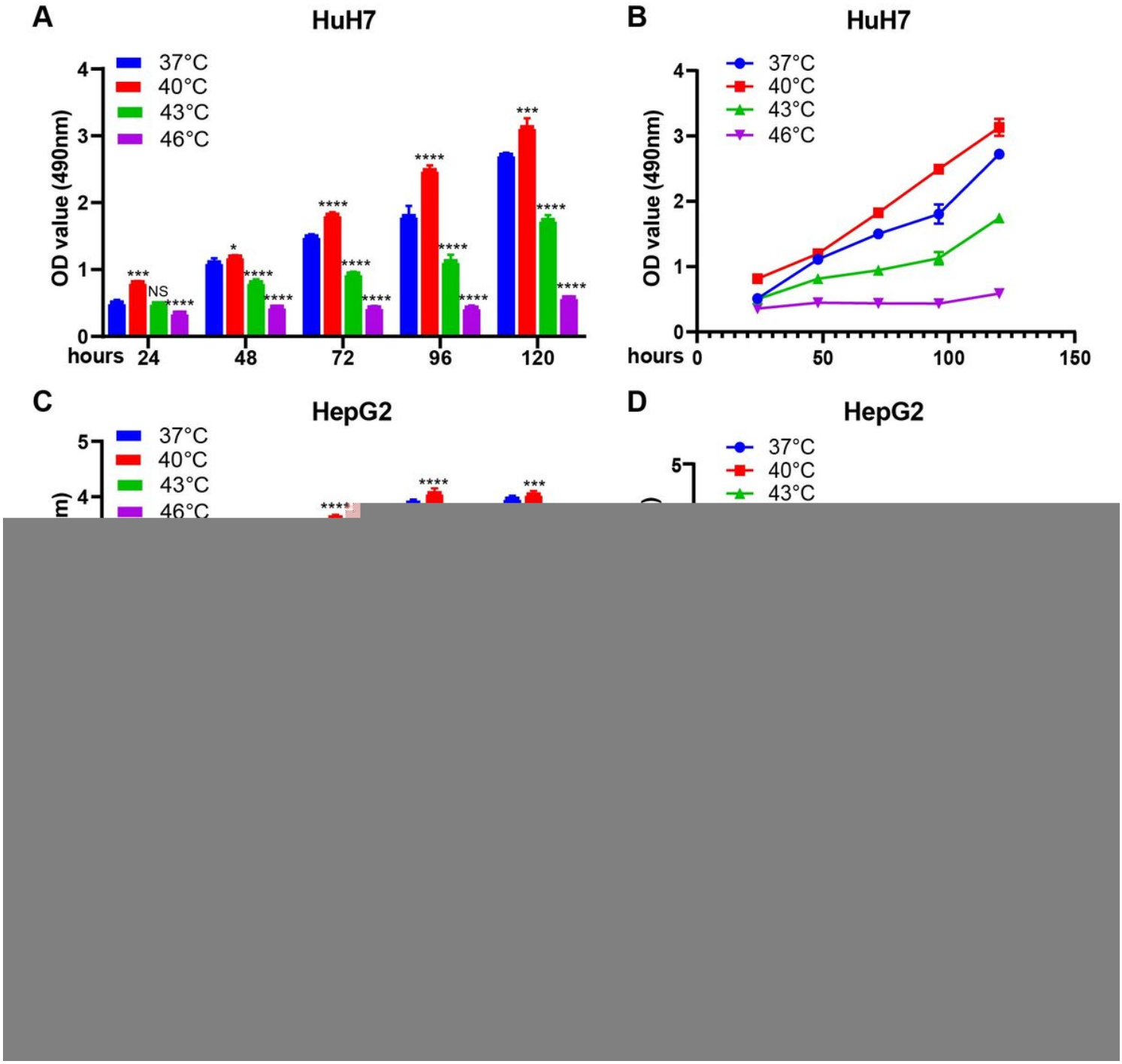

Figure 1

Hyperthermia inhibited the proliferation of hepatoma cells *in vitro*

(A, B, C, D) Hepatoma cells were heat treated at different temperatures (40 ℃, 43 ℃ and 46 ℃), and the cell proliferation was evaluated by CCK-8 assay. (E, F) Plate cloning assay was used to analyze the colony growth of hepatoma cells HepG2 and Huh7 after heat treatment at 43 ℃.

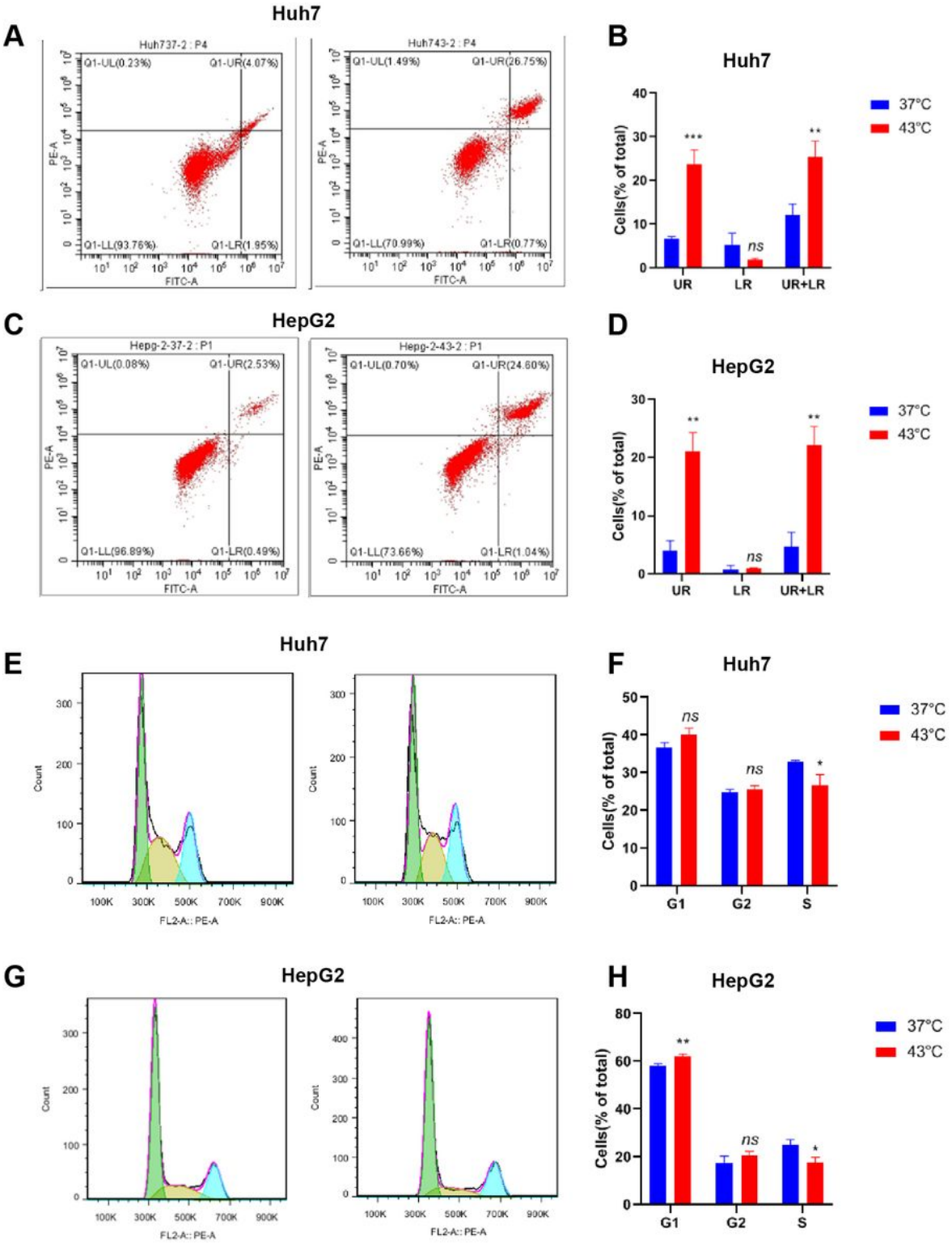

Figure 2

Hyperthermia inhibited the proliferation of liver cancer cells and promoted apoptosis

(A, C) Annexin V / PI double staining was used to detect the effect of hyperthermia on the apoptosis rate of hepatoma cells HepG2 and Huh7. (E, G) The effect of hyperthermia on the cycle distribution of HepG2 and Huh7 cells was measured by propidium iodide staining. (B, D) The histogram describes the

percentage distribution of apoptotic cells, and all data represent three independent experiments. (F, H) The histogram describes the percentage distribution of cell cycle, and all data represent three independent experiments.

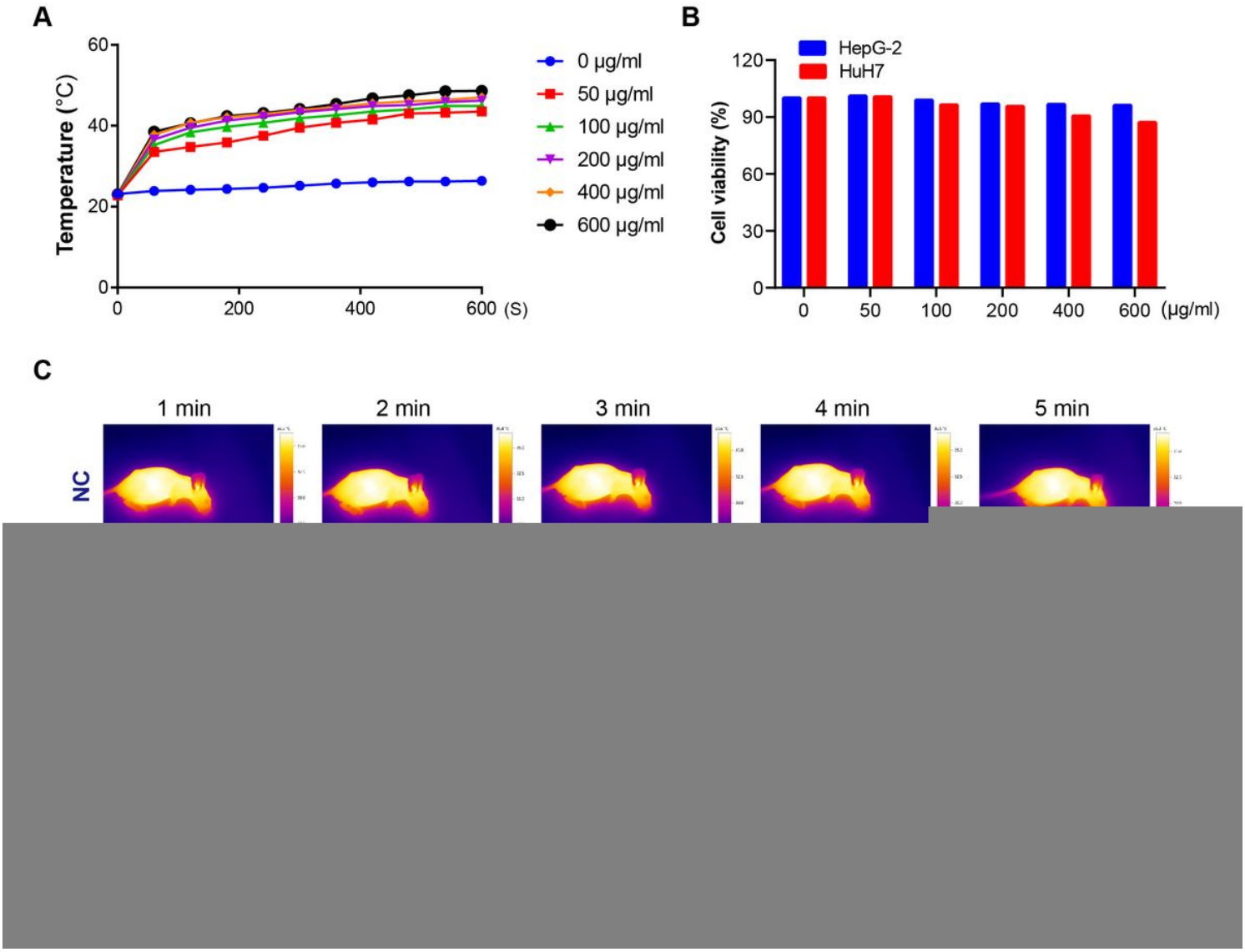

Figure 3

Photothermal material ICG has good heating effect and low cytotoxicity

(A) Temperature change curve of ICG aqueous solution with different solubility under infrared light irradiation of 808nm ,1W/cm2 for a period of 600s. (B) CCK-8 assay was used to detect the cytotoxicity of different concentrations of ICG aqueous solution to liver cancer cells. (C) Near infrared thermography of mice.

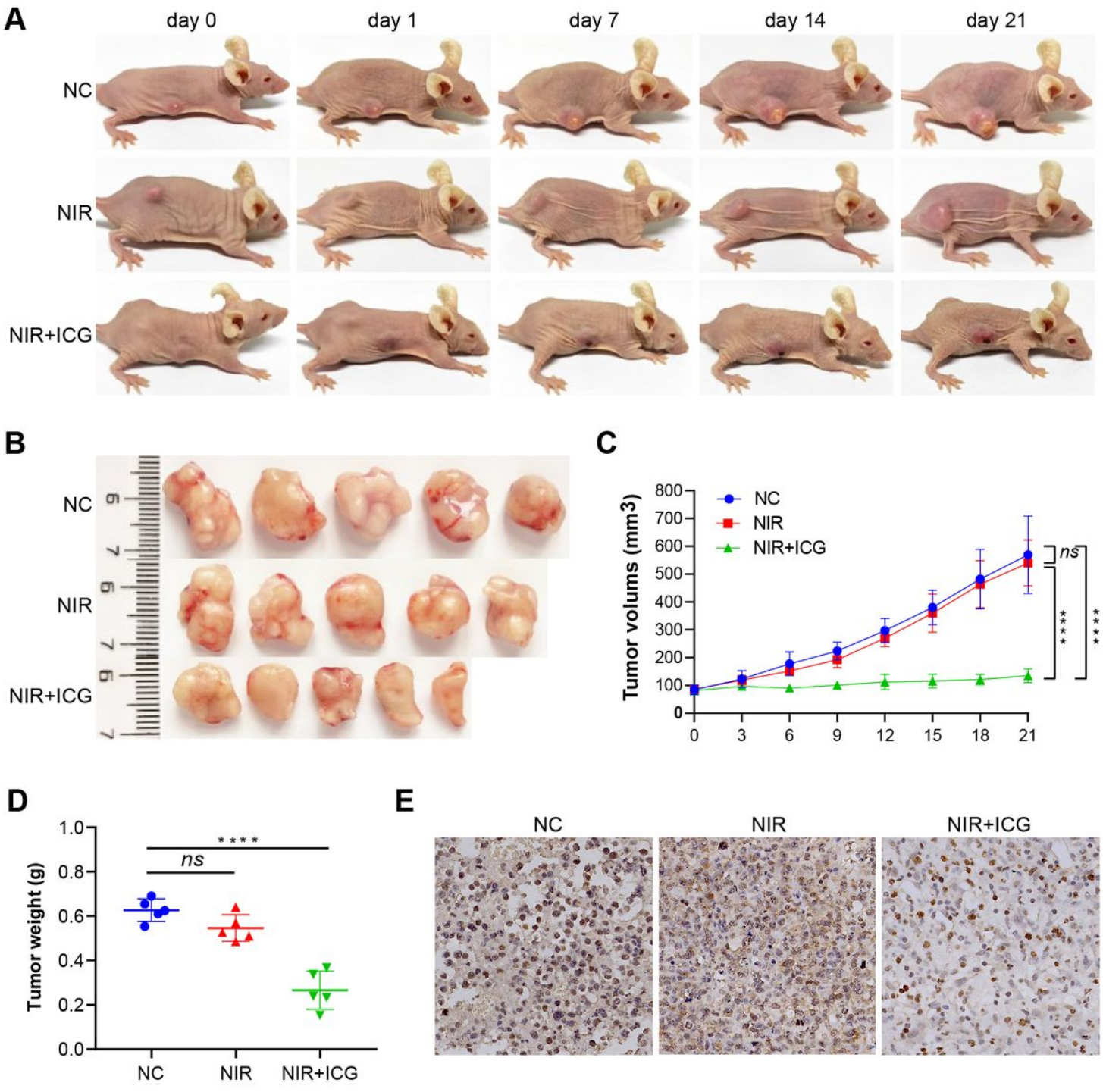

Figure 4

Hyperthermia inhibited the growth of liver cancer in vivo

(A) Representative photographs of HepG2 tumor-bearing nude mice during 0-21 days treatment. (B) Photograph of tumors after excision from different groups of mice at the end of treatments. (C)The tumor volumes of HepG2 tumor-bearing mice were measured on the indicated days. (D) The final tumor weights of HepG2 tumor-bearing mice were measured. (E) Histopathological analyses of tumor growth in mice

xenografted with HepG2cancer cells. The tumor sections were subjected to IHC staining using an antibody against Ki-67. The magnification is 200x.

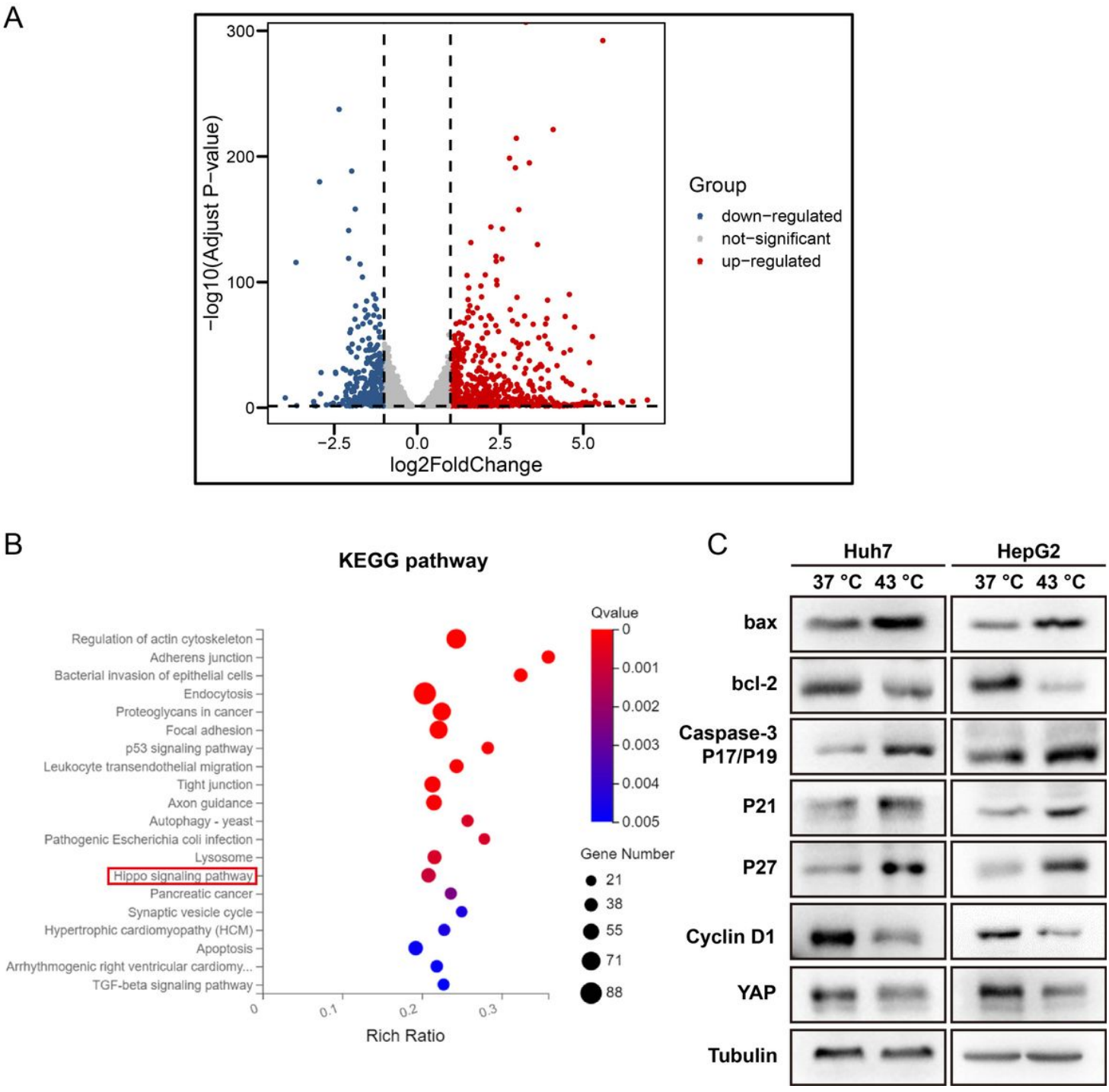

Figure 5

Local hyperthermia can regulate Hippo signaling pathway

(A) RNA-seq showed up-regulated and down-regulated gene changes. (B) KEGG pathway analysis of differential gene changes in up-regulated signal pathway. (C) WB results showed that compared with the

control group, YAP, CyclinD1 and bcl-2 were significantly down-regulated, and bax, Caspase-3p17/p19, P21 and P27 were up-regulated in HCC cells after heat treatment.

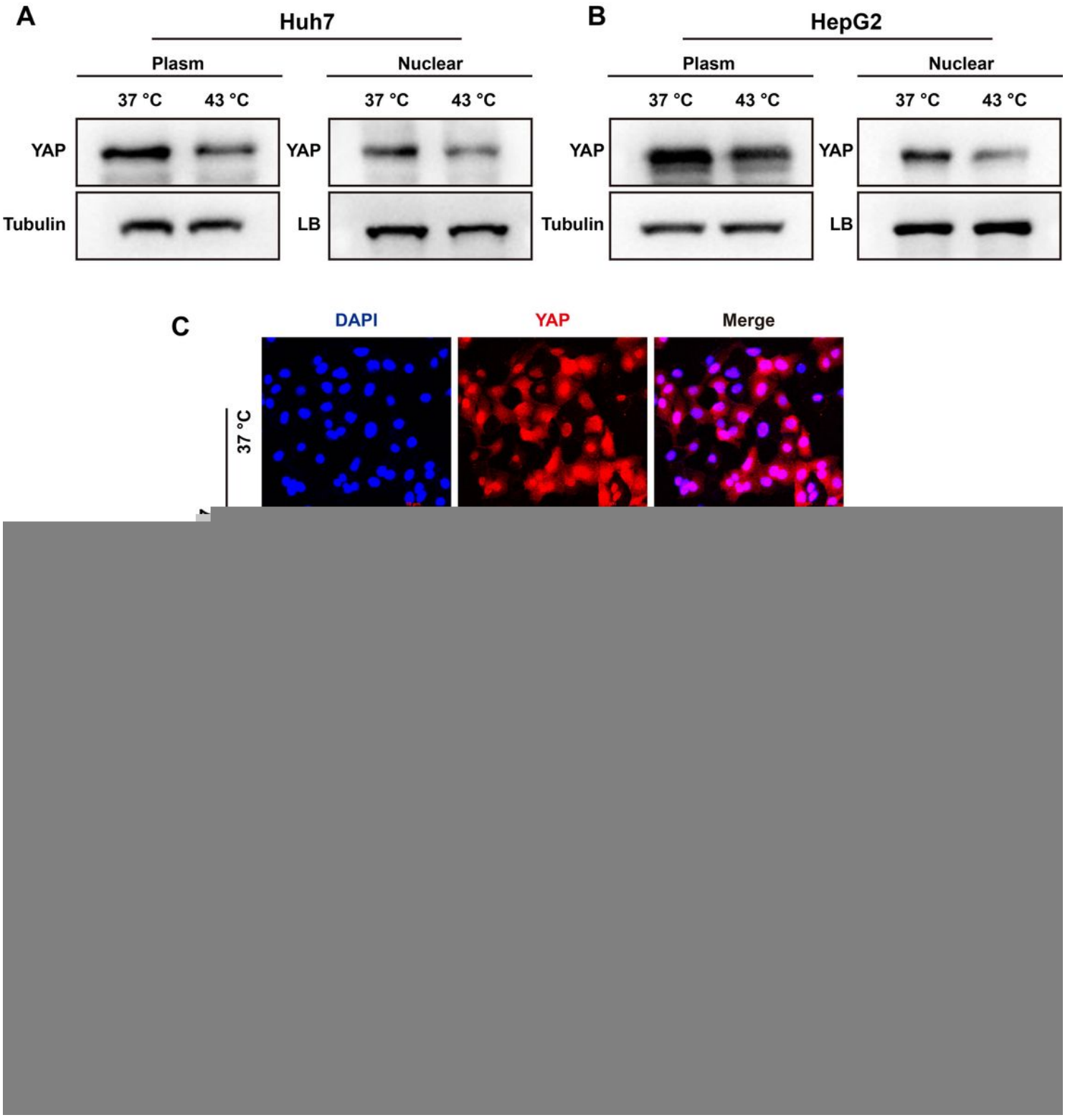

Figure 6

Local heat therapy can inhibit the expression of YAP protein in the nucleus of hepatocellular carcinoma.

(A, B) The results of nucleocytoplasmic separation showed the expression of YAP in the nucleus and cytoplasm of hepatocellular carcinoma after hyperthermia. (C) Immunofluorescence was used to analyze the nuclear localization of YAP protein in hepatocellular carcinoma cells after hyperthermia.