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NFκB mediated elevation of KCNJ11 promotes tumor progression of hepatocellular carcinoma through interaction of lactate dehydrogenase A

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ABSTRACT

It has been well documented that changes in ion fluxes across cellular membranes is fundamental in maintaining cellular homeostasis. Dysregulation and/or malfunction of ion channels are critical events in the pathogenesis of diverse diseases, including cancers. In this study, we focused on the study of K^+ channels in hepatocellular carcinoma (HCC). By data mining TCGA cohort, the expression of 27 K^+ channels was investigated and KCNJ11 was identified as a key dysregulated K^+ channels in HCC. KCNJ11 was differentially expressed in HCC and predicted a poor prognosis in HCC patients. Inhibition of NFκB signaling suppressed KCNJ11 expression in HCC cells. Knockdown of KCNJ11 expression inhibited cell proliferation, promoted cell apoptosis, and reduced cell invasive capacity. Mechanistically, we found that KCNJ11 promotes tumor progression through interaction with LDHA and enhancing its enzymatic activity. Pharmacological inhibition of LDHA largely compromised the oncogenic function of KCNJ11 in cell proliferation, cell apoptosis, and cell invasion. Collectively, our data, as a proof of principle, demonstrate that KCNJ11 acts as an oncogene in HCC though forming a complex with LDHA and suggest that targeting KCNJ11 can be developed as a candidate tool to dampen HCC.

1. Introduction

Over the past decade ion channels have been demonstrated to be aberrantly expressed in cancer cells and contribute to multiple malignant phenotypes of cancer cells, such as unlimited proliferation, metabolic reprogramming, apoptosis resistance, drug resistance, stimulation of neo-angiogenesis, and cell migration and invasiveness [1–3]. Ca^{2+}, Na^+, K^+, and Cl^- channels are essential regulators of cell proliferation and tumor progression [4–8]. Interestingly, many ion channel modulators are FDA-approved drugs and available in clinical use [9,10]. Therefore, ion channels are proposed as potential targets in cancer treatment.

K^+ channels have the greatest amount of diversity among ion channels in the plasma membrane. K^+ channels can crosstalk with multiple cellular signaling cascades and regulate a wide range of physiologic processes by adjusting the intracellular K^+ concentration, creating the membrane potential, regulating cell volume, and enhancing Ca^{2+} signaling [1]. Many studies have shown that expression of certain channels correlating with cancer stage and ectopic expression of K^+ channels occurs in human cancers [11–14].

Based on their activation mechanisms and domain structure, K^+ channels can divided into four main classes: calcium-activated K+ channels (KCa), voltage-gated K^+ channels (Kv), inward-rectifier K^+ channels (Kir), and two-pore-domain K^+ channels (K2P). KCNJ11 is an integral membrane protein and inward-rectifier type potassium channel [15,16]. Mutations in KCNJ11 can lead to familial persistent hyperinsulinemic hypoglycemia of infancy (PHHI), an autosomal recessive disorder characterized by unregulated insulin secretion [17]. Defects in KCNJ11 may also contribute to autosomal dominant non-insulin-dependent diabetes mellitus type II (NIDDM),

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transient neonatal diabetes mellitus type 3 (TNDM3), and permanent neonatal diabetes mellitus (PNDM) [18]. KCNJ11 plays an important role in the regulation of insulin secretion by β cells and pathogenesis of diabetes mellitus [19]. However, little is known about its roles in cancers.

In the present study, we fully addressed the expression profile of K^+ channel genes in hepatocellular carcinoma (HCC). As a result, KCNJ11 was identified as a key dysregulated K^+ channels and was regulated by NFκB signaling in HCC. We then confirmed the oncogenic roles of KCNJ11 in cell proliferation, cell apoptosis, and cell invasion by loss-of-function study and gain-of-function study. Finally, we validated the interaction between KCNJ11 and LDHA by co-immunoprecipitation and showed that LDHA partially mediates the pro-tumor effects of KCNJ11 in HCC.

2. Materials and methods

2.1. Cell culture and reagent

The human HCC cell lines Hep3B, MHCC-97H, MHCC-97L, Huh7, SUN-423, and HepG2 were purchased from Shanghai Institute of Cell Bank. The non-malignant THLE-2 cells were preserved in our hospital. Cells were all cultured in DMEM (Gibico) medium containing 10% fetal bovine serum (FBS, Gibco, USA), 1% streptomycin-penicillin (Sigma, Shanghai, China), 2 mM glutamine, and 10 mM HEPES buffer at 37 °C in a 5% CO_2 atmosphere. The LDHA inhibitor FX11 and pathway inhibitors (LY294002, U0126, Rapamycin, JSH-23, XAV-939, and FLI-06) were all purchased from Selleck (Shanghai, China).

2.2. Clinical samples

Tumor tissue samples used in this study were obtained from the Rizhao City People's Hospital. Samples from these patients were collected between 2012 and 2015 and used for quantitative real-time PCR. The commercial HCC tissue microarray (OD-CT-DgLiv01-012) was purchased from Shanghai Outdo Biotech Inc. All patients enrolled were provided with written informed consent and this experimental procedure was approved by the Ethics Committee of Qingdao University.

2.3. Data mining of TCGA

Level 4 gene expression data were downloaded for Liver Hepatocellular Carcinoma (LIHC) from TCGA, which were processed by Broad Institute's TCGA workgroup. The RNA-seq level 4 gene expression data contain upper quartile-normalized and log2-transformed RNA-seq by expectation maximization (RSEM) values summarized at gene level. Specifically, genes expressed in less than 80% of the samples were removed.

2.4. Cell transfection

For transient transfection, cells were plated at 60–70% confluence in 60 mm dishes. Two specific siRNAs target KCNJ11 were synthesized from GenePharma (Shanghai, China). Control siRNA targeting no known gene sequence was used as the negative control. Lipofectamine® RNAiMAX reagent (ThermoFisher Scientific, #13778030) was used to conduct siRNA transfection according to the manufacturer's protocol. For stable transfections, the KCNJ11 coding region was inserted into pcDNA3.1 (GenePharma) and transfected into Hep3B and MHCC-97L cells to stably overexpress KCNJ11. Cells stably transfected with the pcDNA3.1 empty expression vector (Invitrogen) were used as negative control. Positive clones were selected with G418 (Invitrogen, USA).

2.5. RNA extraction and real-time PCR

Total RNA from tissue or cell samples isolated using Trizol (TaKaRa, Dalian, China) according to the manufacturer's instruction. Total RNA (1μg) was converted to cDNA using PrimeScript Reverse Transcriptase (TaKaRa, China). Quantitative real-time PCR was performed on the ABI 7900 Prism HT (Applied Biosystems, USA), followed by melting curve analysis. The $2^{-\triangle\triangle Ct}$ method was used to assess the gene expression fold change among groups. Three independent experiments were performed. The primers used in this study were shown as follows. KCNJ11, 5′-TGATCCTCATCGTGCAGAACA-3′ (forward) and 5′-ACCCACACGTAGCATGAAGC-3′ (reverse); LDHA, 5′-ATGGCAACTCTAAAGGATCAGC-3′ (forward) and 5′-CCAACCCCAACAACTGTAATCT-3′ (reverse); ACTB, 5′-CATGTACGTTGCTATCCAGGC-3′ (forward) and 5′-CTCCTTAATGTCACGCACGAT-3′ (reverse).

2.6. Immunohistochemistry and immunofluorescence

Immunohistochemical staining were performed using routinely described methods. For antibody staining, tissue-containing slides were deparaffinized, rehydrated in an alcohol series, and stained with a primary antibody against KCNJ11 (ab79171; diluted at 1:200, Abcam). To define KCNJ11 expression, samples without any staining as 1 score and other samples were defined as low (2 scores), medium (3 scores), or high (4 scores) levels of expression. The scoring system was evaluated by two independent individuals who were blinded to the slides and the clinical data examined. For immunofluorescence staining, samples of treated cells were stained with anti-KCNJ11 (ab79171; diluted at 1:200, Abcam), followed by incubation with donkey anti-rabbit Alexa Fluor 594 (1:400, Jackson ImmunoResearch, #711-585-152). Positive cells were quantified by confocal microscopy and analyzed by ImagePro Plus software.

2.7. Western blotting

Whole cell extracts were prepared in a lysis buffer, and cellular protein was measured using a BCA protein assay kit (Pierce, Bonn, Germany). Total cellular protein were separated by 10% SDS-PAGE (10% acrylamide) and transferred to a nitrocellulose membrane (Millipore, Billerica, MA, USA). After blocking in 5% skim milk for 1 h, the membranes were incubated with primary antibodies against KCNJ11 (ab79171; diluted at 1:1000, Abcam), LDHA (ab208093; diluted at 1:1000, Abcam), and β-actin (1:1000; A4700, Sigma Aldrich) overnight at 4 °C. After washing three times with TBST, the membrane was incubated with the HRP-conjugated secondary antibody for 1 h at room temperature and proteins bands were visualized using enhanced chemiluminescence (Pierce).

2.8. Cell proliferation, apoptosis, and invasion assay

For cell proliferation assay, the Cell Counting Kit-8 (CCK-8, Solarbio, Beijing, China) was used. HCC cells (3×10^3 per well) seeded in 96-well plates were incubated with for 5 days. At indicated time point, 10 μl of CCK-8 solution was added to each well, and the 96-well plate was incubated at 37 °C for 1 h. The absorbance at 450 nm was measured by a multi-label plate reader (Bio-Rad, Hercules, California USA). For cell apoptosis assay, caspase-3/7 activity assay and Annexin V/PI staining assay were conducted. For caspase-3/7 activity assay, cells were seeded on 96-well plates at a density of 7000 cells per well. After serum starvation for 48 h, cell number and caspase-3/7 activity were monitored on the same sample using CellTiter-Blue (Promega G8081) and Apo-ONE Caspase-3/7 assay (Promega G7790), respectively. The relative Caspase-3/7 activity was estimated as the ratio Apo-ONE/CellTiter-

Blue signals. For Annexin V/PI staining assay, cells were washed with PBS and stained with Annexin V and Propidium Iodide (BD Pharmingen, USA). Apoptotic cells were measured using a FACS-Calibur (BD Biosciences, USA). Annexin V^+/PI^+ (late apoptotic) and Annexin V^+/PI^- (early apoptotic) cells were quantified by the frequency of fluorescently labeled cells. For cell invasion assay, cell invasion was assessed using the Matrigel Invasion Chamber of pore size 8 mm (Corning, UK). In brief, a total of 1×10^4 cells were seeded into the upper chamber pre-coated with matrigel (Sigma). Medium (600 μl) containing 10% FBS was placed in the lower chamber. After incubation for 48 h, the invasive cells at the bottom of the membrane were fixed with 4% paraformaldehyde solution and then counterstained with DAPI, and cells that did not pass through the filter were removed with cotton swabs. The migrated cells were photographed at a magnification of 400 × in five random fields.

2.9. In situ proximity ligation assay

In situ proximity ligation assay (PLA, Olink Bioscience, DUO92007) was performed to investigate the interactions between KCNJ11 and LDHA according to manufacturer's protocol. Primary antibodies against KCNJ11 (ab79171; diluted at 1:100, Abcam) and LDHA (ab208093; diluted at 1:100, Abcam) were used in this experiment. For negative controls, cells were treated with the mouse and rabbit IgG to instead primary antibodies added. Finally, slides were counterstained with DAPI to locate the nuclei.

2.10. Measurement of lactate production

Lactate secretion in the culture medium of HCC cells was detected by using the commercial Lactate Assay Kit (BioVision, Mountain View, CA) in fully accordance to the manufacturer's instruction. Results were normalized on the basis of the total protein concentration of each sample. All the experiments were performed in triplicate and repeated twice.

2.11. Statistical analysis

Data were expressed as mean and SD. Statistical analysis was performed using performed using Prism 5 software (GraphPad). Continuous variables were evaluated using an unpaired Student *t*-test for comparisons between two groups. Survival analysis was conducted using the Kaplan–Meier method with the log-rank test. Two-sided tests were performed with a $P < 0.05$ indicating statistically significant.

3. Results

3.1. Differentially expressed KCNJ11 predicts a poor prognosis in HCC

Potassium (K^+) channels have the greatest amount of diversity among ion channels in the plasma membrane, which are encoded by over 75 distinct genes [2]. To identify the potential differentially expressed K^+ channels in HCC, we compared their expression in HCC tumor and non-tumor tissues in the TCGA cohort. Genes expressed in less than 80% of the samples were removed. As a result, a total of 27 K^+ channels was included and three K^+ channels (KCNJ11, KCNJ4, and KCNQ1) had a fold change larger than 2 (Fig. 1A). By Kaplan-Meier analysis, we found that higher KCNJ11 expression predicts a poor prognosis in TCGA cohort (Fig. 1B). However, no significant correlation was observed in KCNJ4 or KCNQ1 expression with patients' clinical outcome (Fig. 1B). By data mining the GSE14520 cohort, we also found the inverse correlation between KCNJ11 and patients' outcome (Fig. 1C). To further confirm the expression profile of KCNJ11, we measured the KCNJ11 mRNA and protein expression in HCC tissues. By quantitative real-time PCR, we showed that KCNJ11 mRNA level was significantly upregulated in tumor tissues compared with their corresponding non-tumor tissues (Fig. 1D). By immunohistochemical staining, we found that KCNJ11 protein was highly expressed in 60.9% (28/46) tumor tissues analyzed (Fig. 1E). Meanwhile, patients with a higher KCNJ11 expression had a significant poor prognosis (Fig. 1F). Moreover, to determine the reason for elevated KCNJ11, we treated Huh7 and HepG2 cells with inhibitors of several classical oncogenic pathways including PI3K/AKT signaling (LY294002), MAPK signaling (U0126), mTOR signaling (Rapamycin), NFκB signaling (JSH-23), Wnt/beta-catenin signaling (XAV-939), and Notch signaling (FLI-06). As a result, KCNJ11 expression was significantly downregulated by JSH-23 treatment, suggesting that activated NFκB signaling contributed to increased KCNJ11 expression in HCC (Fig. 1G). Taken together, these findings above suggest that KCNJ11 is overexpressed in HCC and predicts a poor clinical outcome in patients with HCC.

3.2. KCNJ11 knockdown suppresses HCC cell proliferation, apoptosis-resistance, and migration in vitro

To determine the potential oncogenic roles of KCNJ11 in HCC, we performed loss-of-function study. By quantitative real-time PCR, we found that KCNJ11 was commonly and highly expressed in HCC cells as compared to the non-malignant THLE-2 cells (Fig. 2A). Then two cell lines, Huh7 and HepG2, with higher KCNJ11 expression were selected for loss-of-function study. As revealed by immunofluorescence, two siRNAs targeting KCNJ11 resulted in marked decrease in KCNJ11 protein level in both Huh7 and HepG2 cells (Fig. 2B). By CCK-8 assay, we showed that KCNJ11 knockdown significantly inhibited the in vitro cell proliferation of Huh7 and HepG2 cells (Fig. 2C). After serum-starvation for 48 h, we found that si-KCNJ11 cells had remarkable higher caspase-3/7 activity than si-Ctrl cells, indicating the anti-apoptotic role of KCNJ11 in Huh7 and HepG2 cells (Fig. 2D). Similar observation about the effect of KCNJ11 on cell apoptosis was found by Annexin V/PI staining (Fig. 2E). Moreover, genetic silencing of KCNJ11 also suppressed the invasive capacity of Huh7 and HepG2 cells as demonstrated by transwell assay (Fig. 2F).

3.3. KCNJ11 interacts with LDHA

Previously, limited reports are available regarding the mechanism research of KCNJ11. Based on the BioGRID database (https://thebiogrid.org/), we found that KCNJ11 can interact with several proteins including FAM63B, LDHA, and YWHAB. Interestingly, we occasionally found that acidification rate of the si-KCNJ11 cell culture medium was more quickly than that in the si-Ctrl cells, suggesting the interaction between LDHA and KCNJ11 (Fig. 3A). LDHA, a key enzyme regulating aerobic glycolysis, is overexpressed in many human cancers, and correlates with poor clinical outcomes. To evaluate the interaction between KCNJ11 and LDHA in HCC cells, we performed Co-IP assays. Cell lysates from Huh7 and HepG2 cells were immunoprecipitated with an anti-KCNJ11 antibody and probed with anti-LDHA and anti-KCNJ11. As a result, KCNJ11 can form a complex with LDHA (Fig. 3B). Furthermore, the interaction between KCNJ11 and LDHA were confirmed by proximity ligation assay (Fig. 3C). LDHA is the key enzyme of the last step of glycolysis that catalyzes pyruvate to lactate. Consistently, genetic silencing of KCNJ11 led to a significant reduction in lactate production of Huh7 and HepG2 cells (Fig. 3D). Collectively, these data above suggest that KCNJ11 can interact with LDHA in HCC cells.

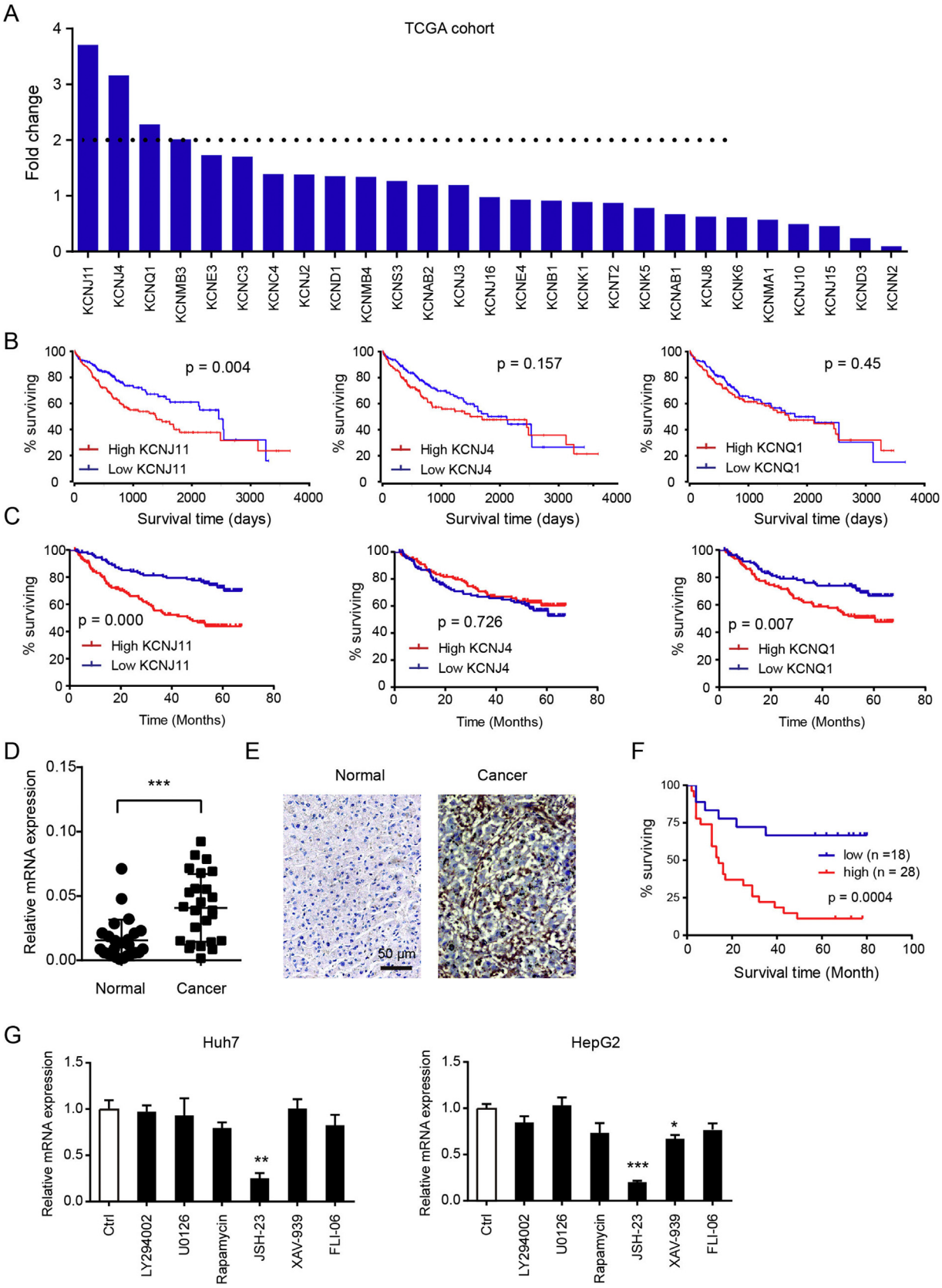

Fig. 1. Differentially expressed KCNJ11 predicts a poor prognosis in HCC. (A) By data mining TCGA cohort, the expression ratio of 27 most abundant K^+ channels in HCC (n = 374) and corresponding normal tissues (n = 51) was shown. (**B-C**) The Kaplan-Meier curves of KCNJ11, KCNJ4, and KCNQ1 in TCGA cohort (**B**) and GSE14520 cohort (**C**). (**D**) The mRNA level of *KCNJ11* in 25 matched liver tumor and non-tumor tissues. **(E)** Representative IHC images of KCNJ11 protein expression in liver tumor and non-tumor tissues. **(F)** The Kaplan-Meier curves of KCNJ11 based on the IHC results. **(G)** The effects of pathway inhibitors on the mRNA expression of *KCNJ11* in Huh7 and HepG2 cells was revealed by real-time qPCR. $^*p < 0.05$; $^{***}p < 0.001$.

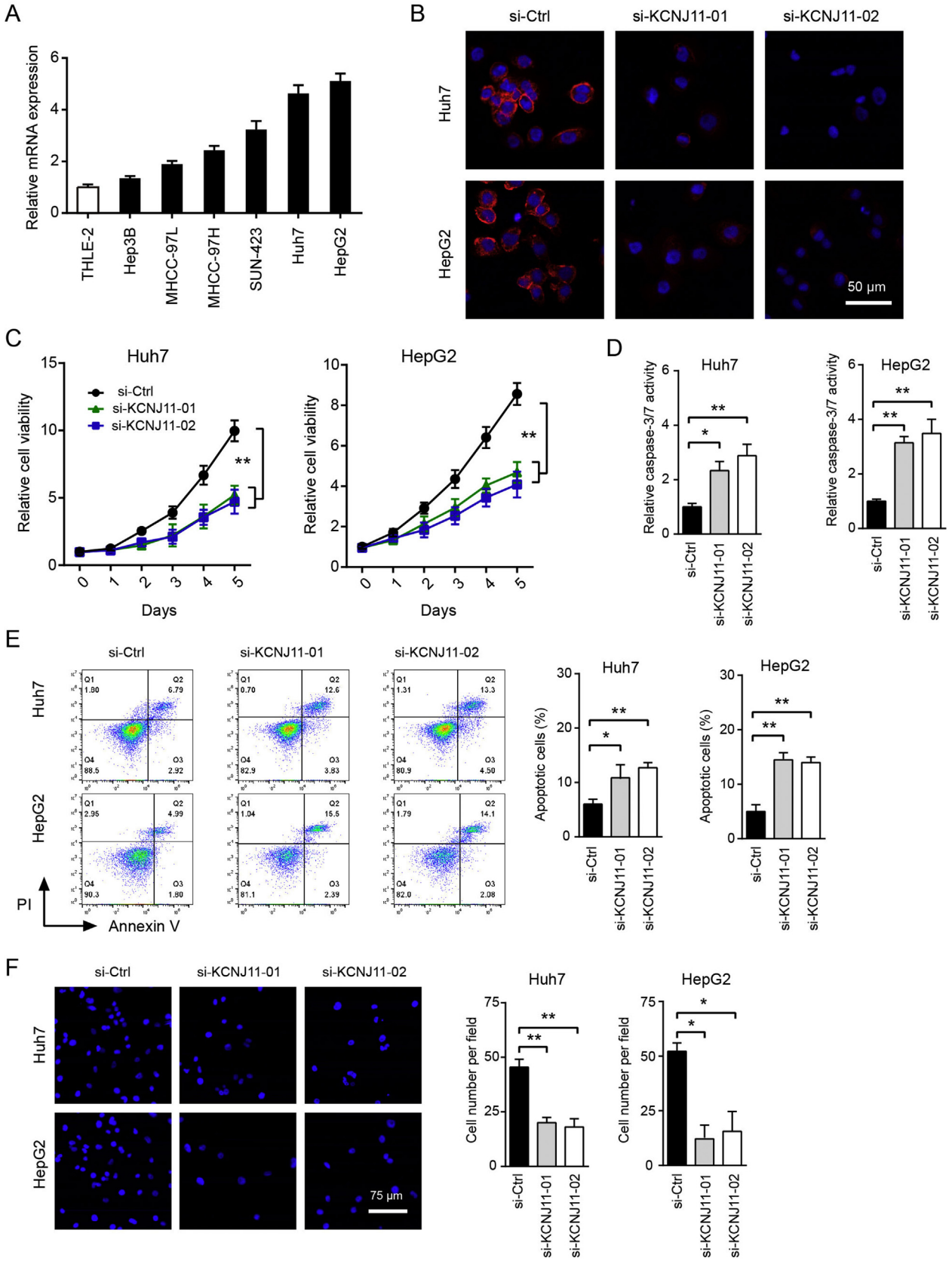

Fig. 2. KCNJ11 knockdown suppresses HCC cell proliferation, apoptosis-resistance, and migration *in vitro*. **(A)** The relative *KCNJ11* mRNA in 6 HCC cell lines and the non-malignant THLE-2 cells was detected by real-time PCR. **(B)** The interference efficiency of two siRNA targeting KCNJ11 in Huh7 and HepG2 cells was measured by immunofluorescence. **(C)** The effects of KCNJ11 knockdown on the cell proliferation of Huh7 and HepG2 cells was determined by CCK-8 assay. **(D–E)** The effects of KCNJ11 knockdown on the cell apoptosis of Huh7 and HepG2 cells was revealed by caspase-3/7 activity and Annexin V/PI staining. **(F)** The effects of KCNJ11 knockdown on the cell invasive capacity of Huh7 and HepG2 cells was detected by transwell assay. $^{*}p < 0.05$; $^{**}p < 0.01$.

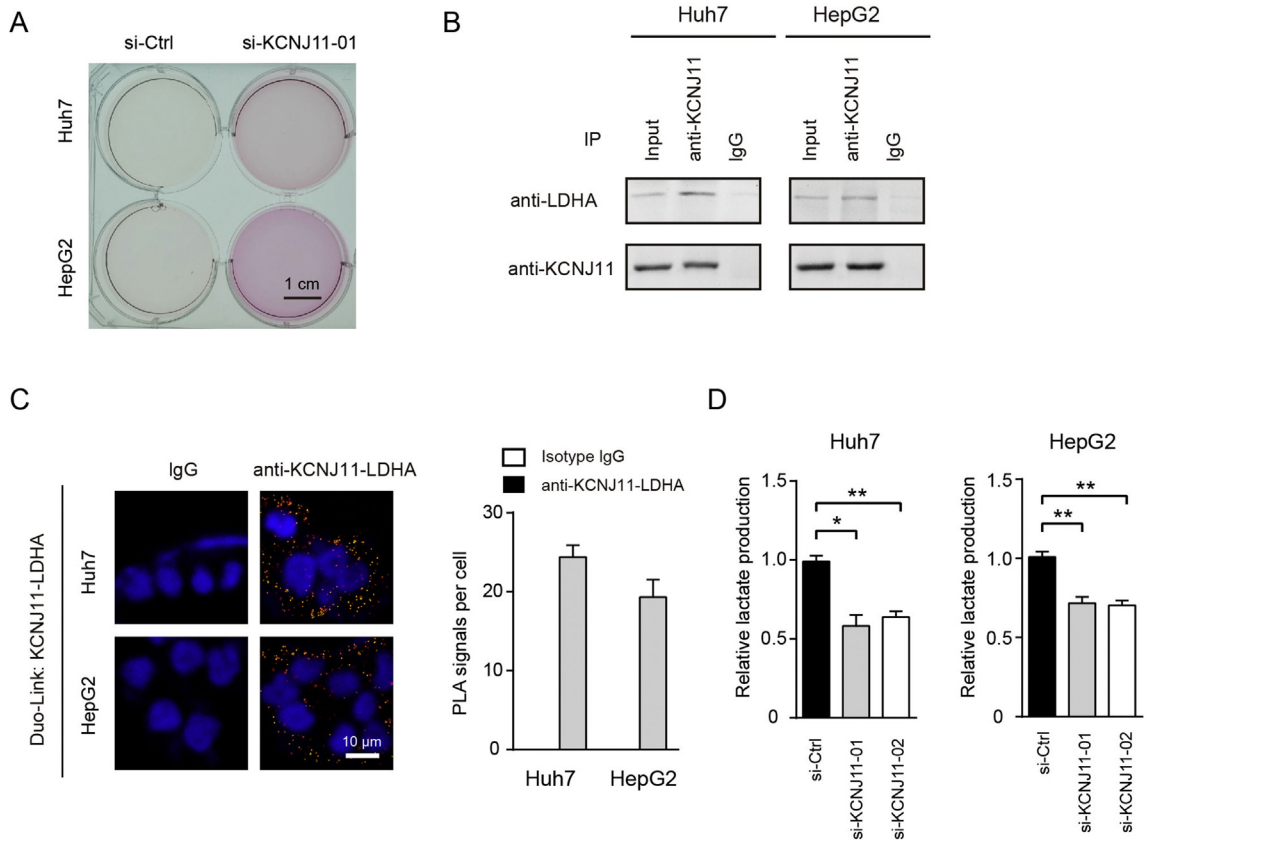

Fig. 3. KCNJ11 interacts with LDHA. (A) The effects of KCNJ11 knockdown on the acidification rate of Huh7 and HepG2 cell culture medium. **(B)** The interaction between KCNJ11 and LDHA was analyzed by Co-Immunoprecipitation (Co-IP). Anti-KCNJ11 antibody was used for IP. The amounts of KCNJ11 and LDHA in the immunoprecipitates were detected by Western blotting with the indicated antibodies. **(C)** The interactions of KCNJ11 and LDHA were studied by in situ proximity ligation assay. Red dots mean positive interaction. **(D)** The effects of KCNJ11 knockdown on the lactate secretion in the culture medium of Huh7 and HepG2 cells. *p < 0.05; **p < 0.01. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. LDHA partially mediated the oncogenic roles of KCNJ11 in HCC

Next, we determined whether LDHA mediates the oncogenic roles of KCNJ11 in HCC cells. By qRT-PCR and Western blotting analyses, we found that KCNJ11 knockdown failed to influence the mRNA (Fig. 4A) or protein (Fig. 4B) level of LDHA in Huh7 and HepG2 cells, but significantly inhibited the LDH activity (Fig. 4C). Therefore, we hypothesized that KCNJ11 might affect the enzyme activity of LDHA and further facilitates tumor progression. To test this hypothesis, we overexpressed KCNJ11 in two cell lines with lower KCNJ11 expression (Fig. 4D) and treated HCC cells with a specific LDHA inhibitor, FX11. As a result, we noticed that overexpression of KCNJ11 promoted cell proliferation (Fig. 4E), inhibited cell apoptosis (Fig. 4F and G), and suppressed cell invasive capacity (Fig. 4G) of Hep3B and MHCC-97L cells. Notably, treatment with FX11 largely compromised the oncogenic roles of KCNJ11 in cell proliferation, cell apoptosis, and cell invasion (Fig. 4E–H). Taken together, these data above suggest that LDHA is critically involved in the oncogenic roles of KCNJ11 in HCC.

4. Discussion

We considerably extend prior works focused on KCNJ11 in cancers. In the current study, we 1) find that elevated KCNJ11 in HCC is mediated by NFκB signaling, 2) show that increased KCNJ11 expression is a prognostic marker conferring worse overall, 3) reveal that KCNJ11 acts as an oncogene in HCC, and 4) demonstrate that KCNJ11 promotes tumor progression by interaction with LDHA.

In the past decade, ion channels have been demonstrated as crucial players in tumor initiation and progression, since dysregulated expression of as well as mutations in several ion channels genes have been uncovered to influence the malignant phenotypes of cancer cells [2,7]. K^+ channels are the most comprehensively characterized regarding their oncogenic activities in facilitating tumor growth and metastasis [20,21]. By comprehensively comparing the expression of K^+ channels in liver tumor and non-tumor tissues, we provided data for a role of Kir6.2, the inward rectifier K^+ channel encoded by KCNJ11, in HCC. Previously, a repertoire of differentially expressed ion channels and transporters during mouse HCC development was identified [22]. And KCNJ11 mRNA expression gradually increased from normal mouse liver tissues to cirrhosis and either pre-neoplastic lesions, and reached peak values in multinodular HCC [22]. Consistently, data derived from TCGA cohort and our cohort further confirm that KCNJ11 expression was upregulated at both mRNA and protein level in human HCC. Single nucleotide repeats in KCNJ11 have been reported to influence colorectal cancer susceptibility [23]. However, no evidence is available regarding the cellular functions of KCNJ11 in cancers. In this study, by both loss-of-function and gain-of-function study, we for the first time revealed that KCNJ11 was critically involved in cell proliferation, cell apoptosis and cell invasion of HCC cells.

Next, we showed a mechanistic interdependence between upregulated KCNJ11 expression and oncogenic function. Our data revealed a novel mechanism that KCNJ11 can interact with LDHA and enhance its enzymatic activity, which further facilitates tumor

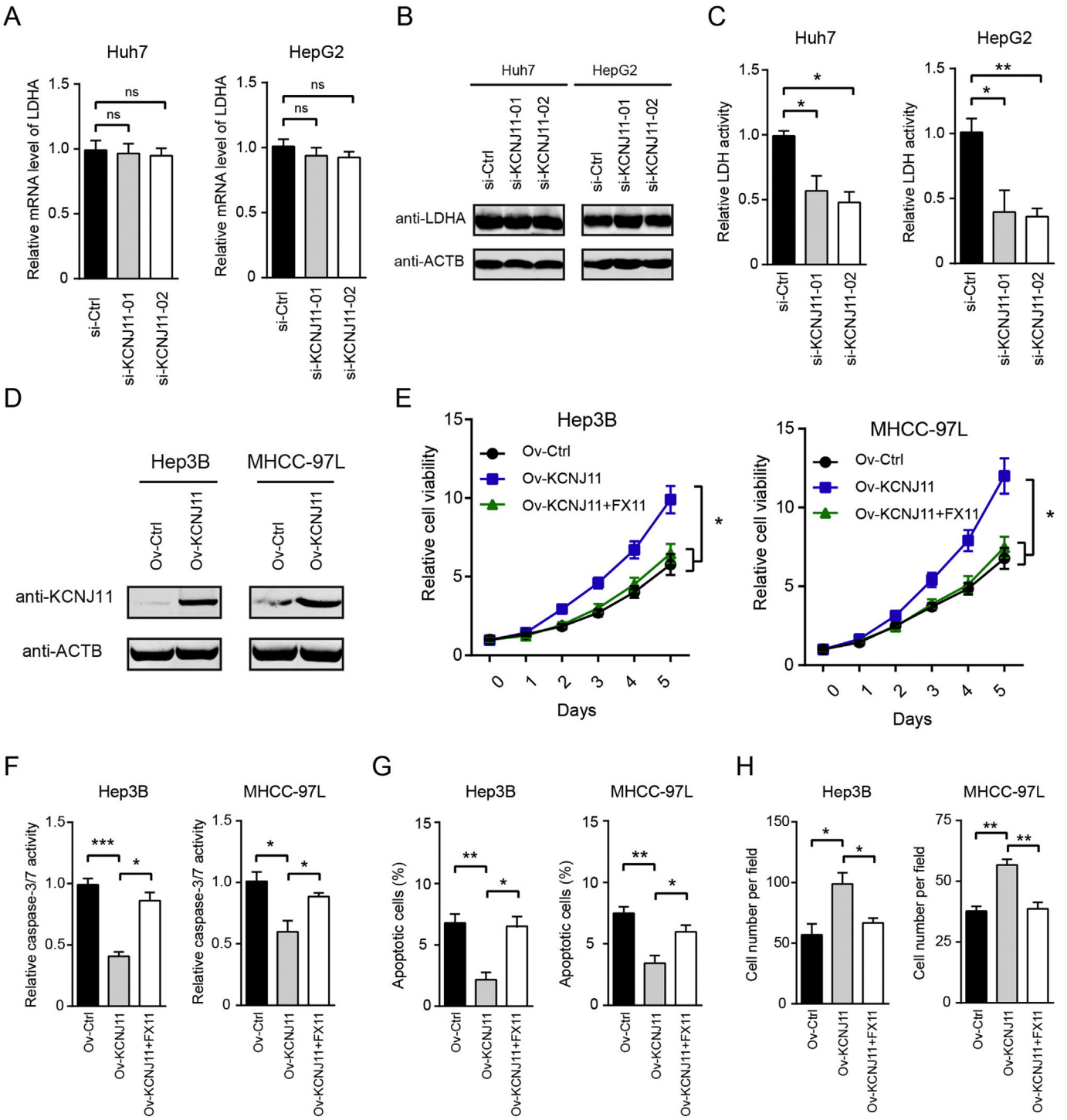

Fig. 4. LDHA partially mediated the oncogenic roles of KCNJ11 in HCC. (A–C) The effects of KCNJ11 knockdown on the mRNA **(A)**, protein level **(B)** and enzymatic activity of LDHA in Huh7 and HepG2 cells. **(D)** The overexpression efficiency of KCNJ11 in Hep3B and MHCC-97L cells was measured by Western blotting. **(E–G)** The effects of KCNJ11 overexpression on the cell proliferation **(E)**, cell apoptosis **(F–G)**, and cell invasive capacity **(H)** of Hep3B and MHCC-97L cells in the absence or presence of LDHA inhibitor FX11. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

progression. In HCC, overexpression of LDHA is correlated poor prognosis and contributes to cell proliferation, cell invasion and glycolysis [24–26]. Knockdown of LDHA significantly attenuated tumor growth and metastasis [27]. In this study, we found that knockdown of KCNJ11 had no significant effect on the mRNA and protein expression of LDHA, but indeed reduced the lactate production of HCC cells. Moreover, pharmacological inhibition of LDHA with FX11 largely compromised the oncogenic activities of KCNJ11 in HCC cells. Apart from the uncovered link between KCNJ11 and LDHA, the possible mechanistic link between the cells resting potential and oncogenic functions in HCC is not well understood and warrants further investigations.

In conclusion, our findings provide evidence that overexpressed KCNJ11 channels predicts a poor prognosis in HCC and that

inhibition of this channel suppresses the proliferation, invasion and promotes apoptosis of HCC cells. KCNJ11 may be a potential and novel target for HCC treatment.

Conflict of interest

The authors declare that there is no conflict of interests.

Acknowledgement

None.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrc.2017.11.011.

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