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Original Articles

HIF-1α-induced RIT1 promotes liver cancer growth and metastasis and its deficiency increases sensitivity to sorafenib

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ABSTRACT

Ras-like-without-CAAX-1 (RIT1) belongs to the RAS superfamily of small GTPases, which plays critical roles in tumor progression. However, little is known about the roles of RIT1 in hepatocellular carcinoma (HCC). Here we found that RIT1 expression was positively associated with the presence of intrahepatic metastasis and the histological grade of HCC and higher RIT1 expression indicated shorter overall survival in HCC patients. *In vitro* and *in vivo* studies revealed that RIT1 functioned as an oncogene, as overexpression of RIT1 enhanced HCC cell proliferation and aggressive behavior, whereas silencing RIT1 expression repressed the malignant behaviors. Furthermore, RIT1 deficiency increased drug sensitivity to sorafenib treatment. We further demonstrated that hypoxia-inducible factor 1α (HIF-1α) directly transcriptionally upregulated RIT1, and its stableness was positively correlated with RIT1 expression in HCC tissues. Knockdown of RIT1 attenuated the invasion and migration induced by hypoxia. Collectively, our data highlight the significance of HIF-1α/RIT1 axis in driving HCC progression and sorafenib resistance.

1. Introduction

Liver cancer is predicted to be the sixth most commonly diagnosed cancer and the fourth leading cause of cancer death worldwide in 2018, with about 841,000 new cases and 782,000 deaths annually [1,2]. Hepatocellular carcinoma (HCC) is the predominate type of primary liver cancer, comprising 75%–85% of cases [1]. HCC is highly resistant to current therapy and 82.4% of patients die within 5 years [3]. Although our understanding of the genetic landscape of HCC has improved significantly through large-scale sequencing studies, the mechanisms that govern HCC progression remains to be explored.

RIT1 (Ras-like-without-CAAX-1) belongs to the RAS superfamily of low molecular weight GTP-binding proteins with significant domain and sequence homology to KRAS, HRAS and NRAS [4–6]. RIT1 contains a well-conserved GTPase core and functions as a guanine nucleotide-regulated molecular switch in the cell by changing between an active GTP-bound and an inactive GDP-bound state [4,5,7]. RIT1 is ubiquitously expressed and its intrinsic GTP hydrolysis activity can be inactivated by Q79L mutation, at the glutamine homologous to Q61 in RAS [8]. When overexpressed, RIT1 Q79L mutant is alone sufficient to transform NIH 3T3 cells through activating MKK3/MKK6-p38γ pathway [9]. In PC6 cells, the activity of RIT1 is induced by stimulation with EGF or NGF and further activates MEK/ERK through direct binding with BRAF [10]. Recently, somatic mutations of RIT1 in ~2% of lung adenocarcinoma cases that cluster in a hotspot near the switch II domain of the protein are reported and ectopic expression of mutated RIT1 robustly induces cellular transformation *in vitro* and *in vivo* [6]. RIT1 is overexpressed and correlates with poor prognosis in endometrial cancer [11]. Moreover, RIT1 abnormalities, including activating mutations and locus amplifications, are significantly more frequent in CMML than other myeloid neoplasms and indicate shorter median overall survival [12].

Although RIT1 possesses intrinsic GTP hydrolysis activity and is most highly homologous with members of Ras subfamily, it displays diverse and complicated biological functions because of its some unique biochemical properties [8]. It has been reported that RIT1 transforms

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NIH3T3 cells in part via RalGDS family proteins, but not the usual kinase cascades of Raf/Mek/MAPK, JNK, p38 or PI3K/AKT [13]. More interestingly, recent study has shown that RIT1 is downregulated in ESCC and significantly associated with poorer prognosis [14]. Further investigation suggests that RIT1 displays tumor-suppressing functions through inhibiting MAPK and PI3K/AKT signaling pathway, EMT, and downregulating cancer stemness of ESCC cells [14].

In HCC, the E81Q somatic *RIT1* mutation has been described in 1 of 50 cases [15]. The amplification of *RIT1* gene is frequently found in HCC (~25%) and might be associated with HCC carcinogenesis and progression [15,16]. However, the biological function of RIT1 in HCC is still unclear. Investigation into the roles of RIT1 and how RIT1 is deregulated in HCC is important for understanding of its molecular mechanism and future development of anti-cancer strategies. In the present study, we demonstrate that RIT1 is associated with HCC progression and promotes HCC cell growth, metastasis and sorafenib resistance. In addition, hypoxia significantly upregulates RIT1 expression in HCC cells via HIF-1α and RIT1 is an important mediator of hypoxia induced migration and invasion.

2. Materials and methods

2.1. Patients samples, tissue microarrays (TMAs) and immunohistochemical analysis

Two hundred and thirty-six human HCC tissue samples were obtained from patients who underwent surgical treatment at the Qidong Liver Cancer Institute (Qidong, China) and the Guangxi Cancer Institute (Nanning, China) between March 2001 and May 2009. The 236 HCC patients included 190 males and 46 females (mean age: 50.86 years, ranging from 21 to 83 years). No patient received preoperative chemotherapy or radiotherapy. All procedures were approved by the Research Ethics Committee of Renji Hospital, Shanghai Jiao Tong University School of Medicine, and informed consent was obtained from each patient. TMAs that included 236 HCC tissues were constructed, and IHC staining, signal evaluation, and statistical data analysis were performed as previously [17]. For signal evaluation of RIT1 and HIF-1α, both the percentage of positive cells (0, ≤10%; 1, 11–25%; 2, 26–50%; 3, 51–75%; 4, > 75%) and intensity of staining (0, no color; 1, light yellow; 2, brownish yellow; 3, brownish black) were considered and the results of IHC staining for RIT1 and HIF-1α were scored 0 to 4 by two independent investigators. The tissues with score of 3 or 4 were defined as high expression and those with score of 0, 1 or 2 were defined as low expression. Antibody information is listed in Supplementary Table 1.

2.2. Cell lines and reagents

Hep3B, HepG2, PLC/PRF/5 and HEK 293T cells were purchased from the American Type Culture Collection (Manassas, VA, USA); Huh7 cells were obtained from the Riken Cell Bank (Tsukuba, Japan); SMMC-7721 cells were provided by the Cell Bank of the Chinese Academy of Sciences (Shanghai, China); MHCC-97L, MHCC-97H and MHCC-LM3 cells were obtained from Zhongshan Hospital, Fudan University (Shanghai, China). HCC-LY10 and HCC-LY5 cells were established from human primary HCC tissues in our laboratory; Li7 and THLE3 cells were purchased from the Shanghai BioLeaf Biotech Company Limited (Shanghai, China). All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and were incubated at 37 °C under a humidified atmosphere with 5% CO_2. Hypoxia (1% O_2) experiments were performed in a NEPCO 1000 hypoxia incubator (Warrensburg, NY). All cell lines were authenticated and characterized by the supplier. Sorafenib, the ERK inhibitor SCH772984 and the AKT inhibitor MK-2206 2HCl were purchased from Selleck Chemicals.

2.3. Vector constructs

The ORF (open reading frame) sequence of RIT1 isoform 1 (Q11), RIT1 isoform 1 (E11) and isoform 2 was PCR amplified using specific primers and cloned into the lentiviral expression vector pWPXL (Addgene, USA). The shRNAs targeting RIT1, HIF-1α and HIF-2α as well as a negative control (NC) were purchased from GeneChem (Shanghai, China). The RIT1 promoter and deletion mutants were generated by PCR and cloned into pGL3-BASIC (Promega, Madison, WI). The fidelities of the constructs were confirmed by sequencing. The information of primers and target sequences is listed in Supplementary Table 2 and Supplementary Table 3.

2.4. Cell counting Kit-8 (CCK-8) assay

Equal numbers of HCC cells were seeded into 96-well culture plates and incubated for 7 days. CCK-8 (Biomake, China) was added to each well and incubated at 37 °C according to the manufacturer's instructions (Biomake, China). After 2 h, the absorbance value was measured at 450 nm. Each experiment was performed in triplicate.

2.5. In vitro plate colony formation assay

Equal numbers of HCC cells were seeded into 6-well culture plates and cultured for 2 weeks. Then cells were fixed in 10% neutral phosphate-buffered formalin and stained with Giemsa (Sigma-Aldrich, St Louis, MO). Each experiment was performed in triplicate.

2.6. In vitro migration and invasion assays

In vitro migration and invasion assays were performed as previously [17]: Equal numbers of HCC cells were seeded into the upper chamber of a transwell (BD Biosciences, Franklin Lakes, NJ, USA) in serum-free media. DMEM containing 10% FBS was added to the bottom chambers. After 24 h or 48 h incubation at 37 °C, cells that had migrated from the upper chamber to the lower chamber were fixed and stained with crystal violet. Cells in five randomly chosen visual fields were counted.

2.7. Tumor xenograft models

For the *in vivo* tumorigenicity and metastasis assay, 1×10^6 HCC cells stably expressing $RIT1^{Q11}$ or pWPXL-control were suspended in 40 μl of a mixture of serum-free DMEM/Matrigel (1:1 vol) for each 6- to 8-week-old male BALB/c nude mouse and orthotopically injected into the left hepatic lobe. After 6 weeks, all animals were sacrificed. The liver and lung tissues were excised and fixed in 10% neutral phosphate-buffered formalin for at least 72 h. H&E staining was performed to detect metastases.

For the experiment to determine the anti-tumor effect of combined treatment with shRIT1 and Sorafenib, NC or shRIT1 expressing HCC-LY10 cells were injected subcutaneously in the right flank of nude mice. When tumors reached a volume of 50 mm^3, mice were randomly assigned to receive sorafenib or vehicle (control) treatment (n = 8). Vehicle and Sorafenib (20 mg/kg/d) were given intraperitoneally five days per week. Tumor volume was measured two times weekly which was calculated by larger diameter × (smaller diameter) ^{2}/2. At day 20 of sorafenib treatment, the mice were euthanized, and the primary tumors were collected and weighed.

All animal experiments were approved by the Shanghai Cancer Institute Experimental Animal Care Commission prior to commencement of the research and complied with the guidelines and regulations of Shanghai Cancer Institute Experimental Animal Care Commission.

2.8. Dual luciferase reporter assay

Cells were seeded into 48-well plates overnight and co-transfected

the relevant reporter plasmids and PRL-TK reporter construct with jetPRIME® DNA/siRNA transfection reagent (Polyplus-transfection® SA, NY, USA). After 48h of incubation, firefly luciferase activity and Renilla activity were detected according to the manufacturer's instructions (Promega, USA).

2.9. Chromatin immunoprecipitation assay (ChIP)

Chromatin immunoprecipitation assay were performed in MHCC-97L and HCC-LY10 cells as previously described [18]. Antibody information is listed in Supplementary Table 1.

2.10. Statistical analysis

The results are presented as the mean ± S.D. of 3 independent experiments and two-group comparisons were analyzed using Student's *t*-test using Graphpad Prism 6 (GraphPad Software, La Jolla, CA, USA). Comparisons among three or more group comparisons were conducted using one-way ANOVA. Correlation analysis was performed between RIT1 and HIF-1α using the Pearson's correlation method. A survival analysis was performed using the Kaplan–Meier method. *Chi-square* test and *t*-test were used to analyze associations between RIT1 expression status and clinical characteristics. $P < 0.05$ was considered significant.

3. Results

3.1. RIT1 is upregulated in HCC tissues and correlates with poor prognosis

To address the association between RIT1 and HCC progression, we measured the RIT1 mRNA expression in 90 pairs of human primary HCC tissues and their matched adjacent non-cancerous liver tissues with qPCR. The results showed that RIT1 mRNA level in HCC tissues was much higher than that in matched non-cancerous liver tissues (Fig. 1A), which was an agreement with the analysis of TCGA cohort (Fig. 1B). In addition, the analysis in TCGA cohort showed RIT1 mRNA level was positively correlated with pathological grade (Fig. 1C) and the Kaplan–Meier survival analysis revealed that HCC patients with higher level of RIT1 had a shorter overall survival (OS) time than did patients with lower level of RIT1 (Fig. 1D).

To further explore the clinicopathological role of RIT1 in HCC progression, the protein expression levels of RIT1 in 236 pairs of HCC tissues and non-cancerous liver tissues were assessed using immunohistochemistry staining (IHC). Out of the 236 cases, 133 (56.36%) HCC tissues showed high RIT1 expression and only 81 (34.32%) non-cancerous liver tissues showed high RIT1 expression (Fig. 1E and F). Further analysis showed that RIT1 expression in HCC tissues was positively associated with the histological grade of HCC and the intrahepatic metastasis status (Table 1). However, there was no correlation between RIT1 expression and other clinicopathological factors (Table 1). Overall, our results suggest that the RIT1 might contribute to HCC progression and metastasis.

3.2. RIT1 accelerates HCC cell proliferation in vitro and in vivo

RIT1 has three splice variants: isoform 1, isoform 2 and isoform 3. RIT1 isoform 2 contains 18 extra N-terminal residues relative to RAS; an additional exon in isoform 1 further extends the N terminus by an additional 17 amino acids; and isoform 3 initiates at a Met downstream of the G1 box and thus lacks a critical component of the nucleotide binding site. The residue numbering in previous studies is based on RIT1 isoform 2 [19]. According to our results of RIT1 with western blotting (Fig. 1G and H), both isoform 1 and isoform 2 were detected in HCC tissues and cells. We analyzed RIT1 mutation status of HCC tissues in TCGA PanCancer cohort (http://www.cbioportal.org) [20,21], which only shows RIT1 isoform 2 mutation status, and the results showed that there was no mutation in RIT1 isoform 2 and E81Q somatic RIT1 mutation [15] wasn't found. The mutation status of RIT1 isoform 1 in 21 HCC cases and white blood cells from 4 heathy volunteers were checked. We found one Q11E RIT1 isoform 1 mutation in 5 HCC cases according to the RIT1 sequence in white blood cells (Supplementary Fig. 1A).

To investigate the roles of $RIT1^{Q11}$, $RIT1^{E11}$ and RIT1 isoform 2 in HCC progression, we stably overexpressed $RIT1^{Q11}$, $RIT1^{E11}$ and RIT1 isoform 2 in SMMC-7721, Li7 and Huh7 HCC cells and knocked down the endogenous expression of RIT1 in MHCC-97L and HCC-LY10 cells using lentiviral infection (Supplementary Figs. 1C and D) according to the endogenous protein and mRNA expressions of RIT1 in HCC cells (Fig. 1H and Supplementary Fig. 1B). CCK8 and colony formation assays showed that all $RIT1^{Q11}$, $RIT1^{E11}$ and RIT1 isoform 2 significantly accelerated HCC cell growth (Fig. 2A and B), while knockdown of RIT1 had an opposite effect on HCC cell growth (Fig. 2C and D). RIT1 can transform NIH3T3 cells to tumorigenicity [6,13]. We also assayed the ability of NIH3T3 and Li7 cells stably expressing $RIT1^{Q11}$, $RIT1^{E11}$ and RIT1 isoform 2 to form colonies in soft agar. All three variants induced colony formation of NIH3T3 and Li7 cells in soft agar (Supplementary Fig. 2A). In addition, knockdown of RIT1 repressed colony formation of MHCC-97L and HCC-LY10 cells in soft agar (Supplementary Fig. 2B). Consistent with these findings, overexpression of $RIT1^{Q11}$ enhanced the tumorigenicity of SMMC-7721 and Li7 cells in xenograft mouse models (Fig. 2E). Western blotting analysis showed that xenografts from $RIT1^{Q11}$-overexpressing cells still maintained a high RIT1 expression level (Fig. 2F).

3.3. RIT1 promotes HCC cell migration and invasion in vitro and metastasis in vivo

RIT1 expression was positively correlated with presence of intrahepatic metastasis, so the effects of RIT1 on the metastatic potential of HCC cells were explored. Transwell migration and invasion assays showed overexpression of $RIT1^{Q11}$, $RIT1^{E11}$ and RIT1 isoform 2 promoted the migratory and invasive abilities of SMMC-7721, Li7 and Huh7 cells (Fig. 3A); whereas knockdown of RIT1 inhibited migration and invasion of MHCC-97L and HCC-LY10 cells (Fig. 3B). To investigate the effect of RIT1 on the metastatic potential of HCC cells *in vivo*, 1×10^6 SMMC-7721 cells stably overexpressing $RIT1^{Q11}$ were injected into the left hepatic lobe of nude mouse. SMMC-7721 cells with pWPXL were used as a control. The mice were sacrificed after 6 weeks, the liver and lung tissues of mice in both the control and $RIT1^{Q11}$-overexpressing groups were observed via H&E staining. The tumors of the $RIT1^{Q11}$-overexpressing group more frequently had invasive growth fronts with irregular tumor borders, whereas those of the control group more often had tumor growth fronts with more regular and less invasive borders (Fig. 3C). The presence of tumor microsatellites was observed in both the $RIT1^{Q11}$-overexpressing and control groups, but liver metastasis (tumors in non-inoculated liver lobules) occurred in eight of the ten $RIT1^{Q11}$-overexpressing mice (80%) and only in one of the ten control mice (10%). In addition, six of the ten mice in the $RIT1^{Q11}$-overexpressing group developed lung metastases (60%). However, only one of the 10 mice in the control group developed lung metastasis (10%) (Fig. 3C).

As other Ras family G-proteins, RIT1 transmits cellular signals to specific effectors, which results in the activation of diverse signaling pathways, including mitogen-activated protein kinase (MAPK) family protein kinases and phosphatidylinositol 3-kinase (PI3K)/AKT [protein kinase B (PKB)] [4]. GTPase activity assay showed that overexpression of $RIT1^{Q11}$, $RIT1^{E11}$ and RIT1 isoform 2 enhanced GTPase activities in Li7 and Huh7 cells (Supplementary Fig. 3A). Western blotting analysis of phospho-ERK (p-ERK) and phospho- AKT (p-AKT) revealed that RIT1 increased the levels of p-ERK and p-AKT (Fig. 3D and Supplementary Fig. 3B). Based on the possibility that crosstalk exists between the AKT and ERK signaling pathways under RIT1 regulation, RIT1-overexpressing HCC cells were treated with MK2206 2HCl, a highly

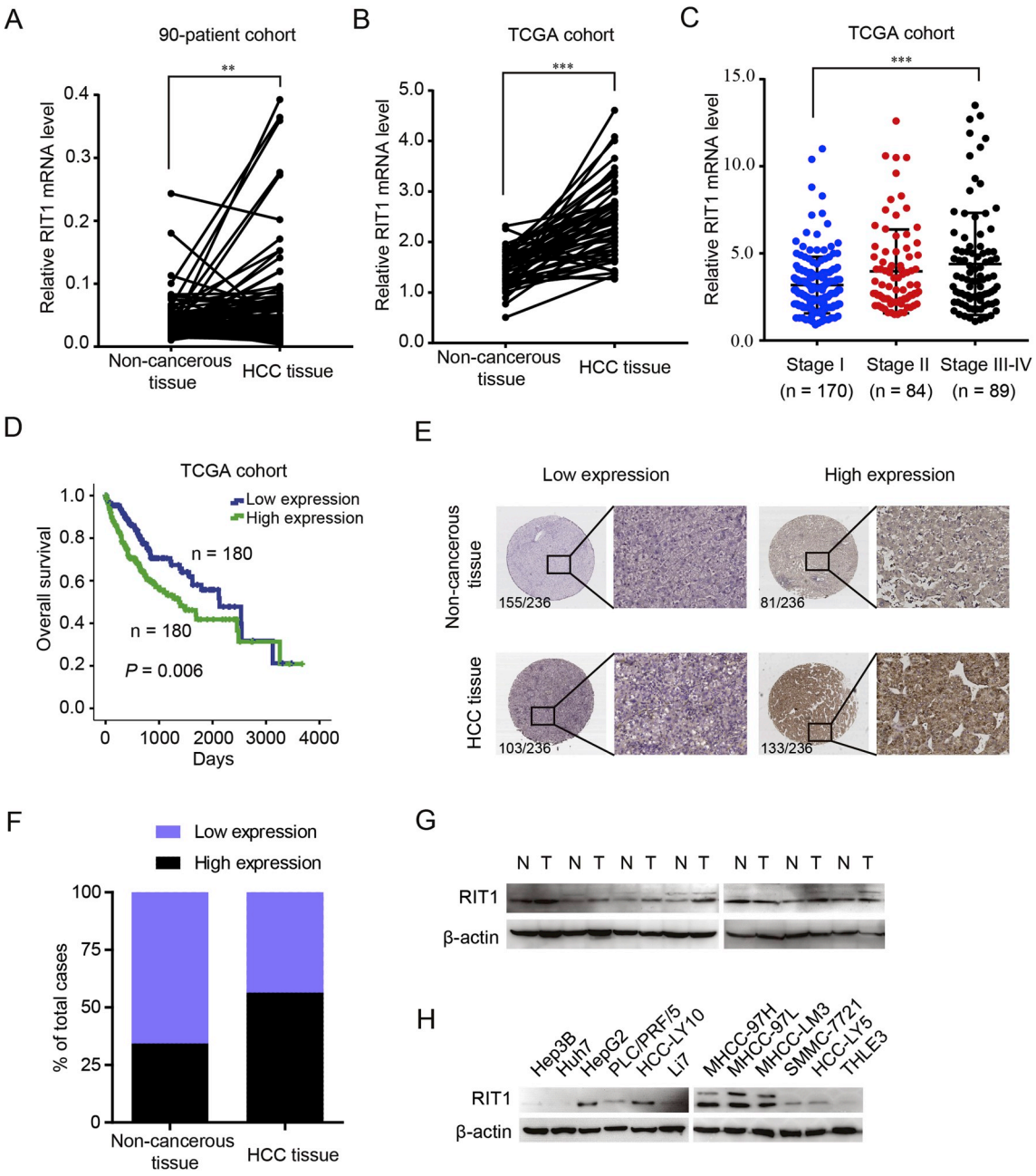

Fig. 1. RIT1 is overexpressed in human HCCs and correlates with poor prognosis. (A) The mRNA levels of RIT1 in 90 pairs of HCC tissues and matched adjacent non-cancerous liver tissues from our lab. (B) The mRNA levels of RIT1 in HCC tissues and matched adjacent non-cancerous liver tissues from TCGA cohort (n = 50). (C) RIT1 expression levels were positively correlated with pathological grade. (D) The Kaplan–Meier analysis revealed the association of RIT1 with the overall survival of HCC patients. (E) Immunohistochemistry staining showed the protein expression of RIT1 in HCC tissues and non-cancerous liver tissues (original magnification: left × 40, right × 400). (F) Bar chart revealed the percentage of RIT1 low/high expression as measured by IHC in HCC tissues and non-cancerous liver tissues. (G) Western blotting analysis of RIT1 protein levels in human primary HCC tissues (T) and the corresponding adjacent non-cancerous liver tissues (N). (H) Western blotting analysis of RIT1 protein levels in 11 HCC cell lines and THLE3 (an immortalized normal human liver epithelial cell line). β-actin was used as a loading control. $^{**}P < 0.01$, $^{***}P < 0.001$.

selective inhibitor of AKT1/2/3, or SCH772984, a specific inhibitor of ERK1/2. It was found that MK2206 2HCl treatment resulted in a marked decrease in p-AKT levels but did not affect the levels of p-ERK. Similarly, treatment with SCH772984 inhibited p-ERK activation and had no effect on the levels of p-AKT (Supplementary Figs. 3C and D). Thus, in RIT1-overexpressing HCC cells, no crosstalk existed between the AKT and ERK signaling pathways.

Taken together, RIT1 plays an important role in HCC cell proliferation and metastasis.

3.4. RIT1 deficiency confers sensitivity to sorafenib in HCC cell lines

Sorafenib is a multikinase inhibitor that is approved as the standard therapy for advanced HCC patients, but it only provides limited survival benefit, especially for Asia-Pacific patients [22,23]. We found that RIT1 was upregulated at both mRNA and protein levels when HCC cells were treated with sorafenib (Fig. 4A and B). MAPK signaling is responsible for the poor response to sorafenib [24], so the effect of RIT1 on sorafenib sensitivity were explored. We analyzed the half maximal inhibitory concentrations (IC50s) of sorafenib in MHCC-97L and HCC-

Table 1
Correlation between RIT1 expression levels in HCC patients and their clinicopathologic characteristics.

Clinical pathology	RIT1 expression		*P* value
	Low n (%)	High n (%)	
Gender			
male	89 (86.41)	101 (75.94)	0.044*
female	14 (13.59)	32 (24.06)	
Age			
≤50	53 (51.46)	68 (51.52)	0.993
> 50	50 (48.54)	64 (48.48)	
AFP (ng/ml)			
≤20	33 (33.33)	46 (34.59)	0.842
> 20	66 (66.67)	87 (65.41)	
HBV infection			
absent	15 (15.46)	27 (20.45)	0.335
present	82 (84.54)	105 (79.55)	
Tumor size (cm)			
< 3	20 (19.80)	21 (16.41)	0.801
3-5	31 (30.69)	41 (32.03)	
> 5	50 (49.51)	66 (51.56)	
Histological grade			
I-II	64(62.14)	55 (41.35)	0.002*
III-IV	39 (37.86)	78 (58.65)	
Intrahepatic metastasis			
absent	78 (75.73)	83 (62.41)	0.029*
present	25 (24.27)	50 (37.59)	
Cirrhosis			
absent	16 (15.53)	22 (16.54)	0.835
present	87 (84.47)	111 (83.46)	

P value represents the probability from a χ^2 test for RIT1 expression levels between variable subgroups. AFP, alpha-fetoprotein. * indicates $P < 0.05$.

LY10 cells. These HCC cells had an IC50 for sorafenib of over 10 μM (Fig. 4C), which indicates that MHCC-97L and HCC-LY10 cells are insensitive to sorafenib. Interestingly, knockdown of RIT1 sensitized HCC cells to sorafenib treatment (Fig. 4C). CCK8 assays also showed that combination of RIT1 knockdown and sorafenib treatment had stronger inhibitory effects on cell growth compared with mono treatment in MHCC-97L and HCC-LY10 cells (Fig. 4D).

Next, we confirmed the above results *in vivo* by using a xenograft model. Our results revealed that RIT1 knockdown or sorafenib (20 mg/kg/day) alone inhibited liver tumor growth *in vivo*, while knockdown of RIT1 expression in HCC-LY10 cells significantly enhanced the efficacy of tumor growth inhibition by sorafenib (Fig. 4E–G). These results provide persuasive evidence that targeting RIT1 signaling is an efficient strategy to enhance the anti-tumor efficacy of sorafenib.

3.5. HIF-1α mediates hypoxia-induced RIT1 overexpression in HCC cells

As shown above, RIT1 is upregulated in HCC tissues and plays important roles in HCC progression and sorafenib resistance. Therefore, knowledge of the underlying the regulation mechanism of RIT1 has major importance and might provide novel insights for the therapy of HCC. Previous studies have shown that amplification of *RIT1* gene is detected in 11 of 43 qualified HCC cases and *RIT1* gene amplification indicates shorter survival time [16]. We also analyzed *RIT1* DNA copy number variation (amplification) in TCGA (The Cancer Genome Atlas) PanCancer cohort using Oncomine (https://www.oncomine.org/resource) and the results showed that *RIT1* DNA copy number's fold change is 1.323 with gene ranking 2 (Supplementary Fig. 4) and there were 42 of 367 (11.44%) HCC cases existing *RIT1* gene amplification (http://www.cbioportal.org) [20,21]. These data indicate that only some RIT1 overexpression is likely due to high level amplification in HCC.

Hypoxia is an important environmental factor in HCC and commonly promotes invasion, metastasis and malignancy [25]. From qPCR and western blotting analysis of four HCC cell lines, SMMC-7721, Li7, MHCC-97L and HCC-LY10, exposed to 20% O_2 (normoxia) and 1% O_2 (hypoxia), it was observed that RIT1 was induced by hypoxia (Fig. 5A and B). Hypoxia-inducible factors (HIFs) are master regulators of oxygen homeostasis, consisting of the constitutively expressed HIF-1β subunit and the O_2-sensitive HIF-1/2α subunit [26,27]. To investigate whether RIT1 is regulated by HIFs, we examined RIT1 mRNA expression in HIF-1α and HIF-2α knockdown HCC cells and found that the hypoxia-induced RIT1 expression was abrogated upon knockdown of HIF-1α but not HIF-2α in MHCC-97L and HCC-LY10 cells (Fig. 5C and D).

3.6. RIT1 is a direct target of HIF-1α

To confirm whether RIT1 is a direct transcriptional target of HIF-1α, RIT1 promoter region from −1231 bp to +636 bp to the first ATG was cloned and four putative hypoxia-responsive elements (HREs) containing HIF-1α-binding consensus sequence 5′-A/GCGTG-3′ were located (Fig. 6A). According to the HRE locations, four truncation mutants were constructed and the results of dual luciferase reporter assays showed that RIT1 promoter activity mainly located between −962 bp and −661 bp (Fig. 6B). The relative luciferase activity of the RIT1 promoter was significantly induced by hypoxia and enhanced luciferase activity was reversed by deletion of HRE-3 (between −962 bp and −661 bp) (Fig. 6C). Chromatin immunoprecipitation (ChIP) assay also demonstrated HIF-1α bound to RIT1 promoter at HRE-3 (Fig. 6 D and E). Collectively, these data suggest that RIT1 is a direct transcriptional target of HIF-1α.

Knockdown of RIT1 attenuates the invasion and migration induced by hypoxia and RIT1 expression is positively correlated with HIF-1α in HCC tissues.

To explore whether RIT1 is a functional downstream target of HIF-1α in HCC cells, endogenous expression of RIT1 was knocked down in MHCC-97L and HCC-LY10 cells under hypoxia (Fig. 7A). Transwell migration and invasion assays showed that hypoxia enhanced invasive and migratory capabilities of HCC cells and knockdown of RIT1 attenuated the invasion and migration induced by hypoxia (Fig. 7B). Next, we analyzed the relationship between RIT1 and HIF-1α expression in HCC tissues. The results showed that there was a positive correlation between the RIT1 and HIF-1α protein expression in HCC tissues ($r = 0.291$, $P < 0.0001$; Fig. 7C and D). Taken together, these results suggest that RIT1 is an important mediator of hypoxia-induced migration and invasion in HCC.

4. Discussion

Recent studies have focused on RIT1 mutations in myeloid malignancies and lung adenoma carcinoma [6,12], and ectopic expression of mutated RIT1 induces cellular transformation via PI3K and MEK activation *in vitro* and *in vivo* [6]. More recently, it has been reported that RIT1 wildtype and the disease-associated variants control actin dynamics via complex formation with RAC1/CDC42 and PAK, which result in cell motility [5]. In our present study, we analyzed RIT1 mutation status in TCGA liver cancer cohort and 21 HCC patients from our lab. We found one Q11E RIT1 isoform 1 mutation, which was not included in the highly conserved domains (G1–G5), in 5 HCC cases according to the sequence of white blood cells. Further analysis showed that RIT1 isoform 1 wildtype, Q11E mutant and isoform 2 had same effects on HCC cell proliferation, migration and invasion, which indicates that the additional exon in RIT1 isoform 1 doesn't affect RIT1 function in HCC progression. As previous reports [4,6], RIT1 regulated AKT and ERK activity and enhanced GTPase activity in HCC cells, suggesting that RIT1 function as an oncogene in HCC mainly via guanosine-triphosphatase (GTPase) activity and the highly conserved domains (G1–G5).

We found that RIT1 was overexpressed in HCC tissues compared with non-cancerous liver tissues. TCGA liver cancer cohort analysis revealed that high level amplification of RIT1 might result in RIT1

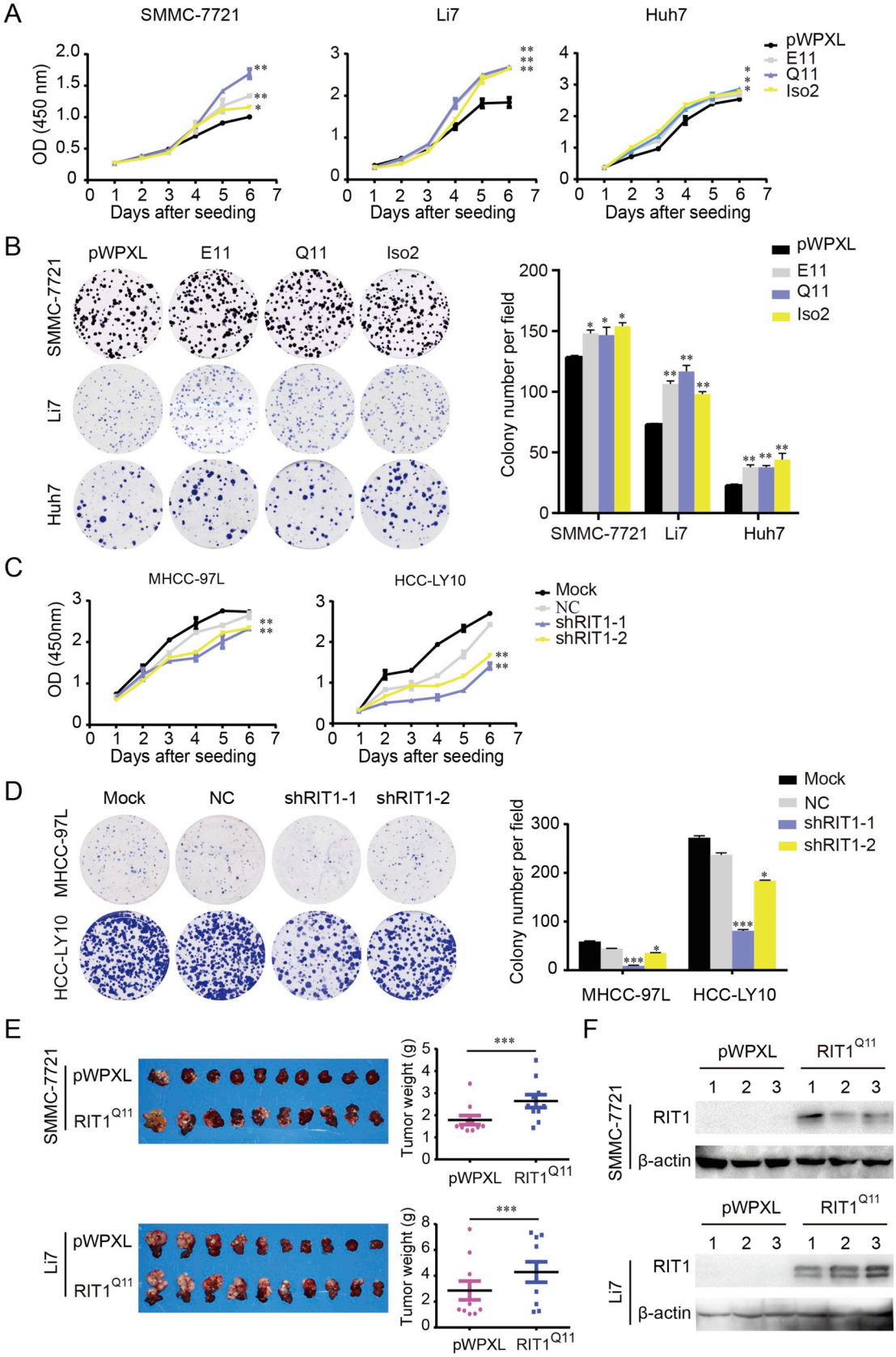

Fig. 2. RIT1 promotes cell proliferation *in vitro* and *in vivo*. (A) Overexpression of RIT1 promoted proliferation of SMMC-7721, Li7 and Huh7 cells by CCK8 assays and (B) clone formation assays. (C) Knockdown of RIT1 inhibited proliferation of MHCC-97L and HCC-LY10 cells by CCK8 assays and (D) clone formation assays. (E) Liver tissues were collected from nude mice with tumor xenografts inoculated with SMMC-7721 (top panel) and Li7 (bottom panel) cells stably overexpressing $RIT1^{Q11}$ or pWPXL (control). Representative images were shown (left) and the livers with xenografts were weighted (right) (n = 10, respectively). (F) The RIT1 protein level was detected by western blotting in xenografts. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

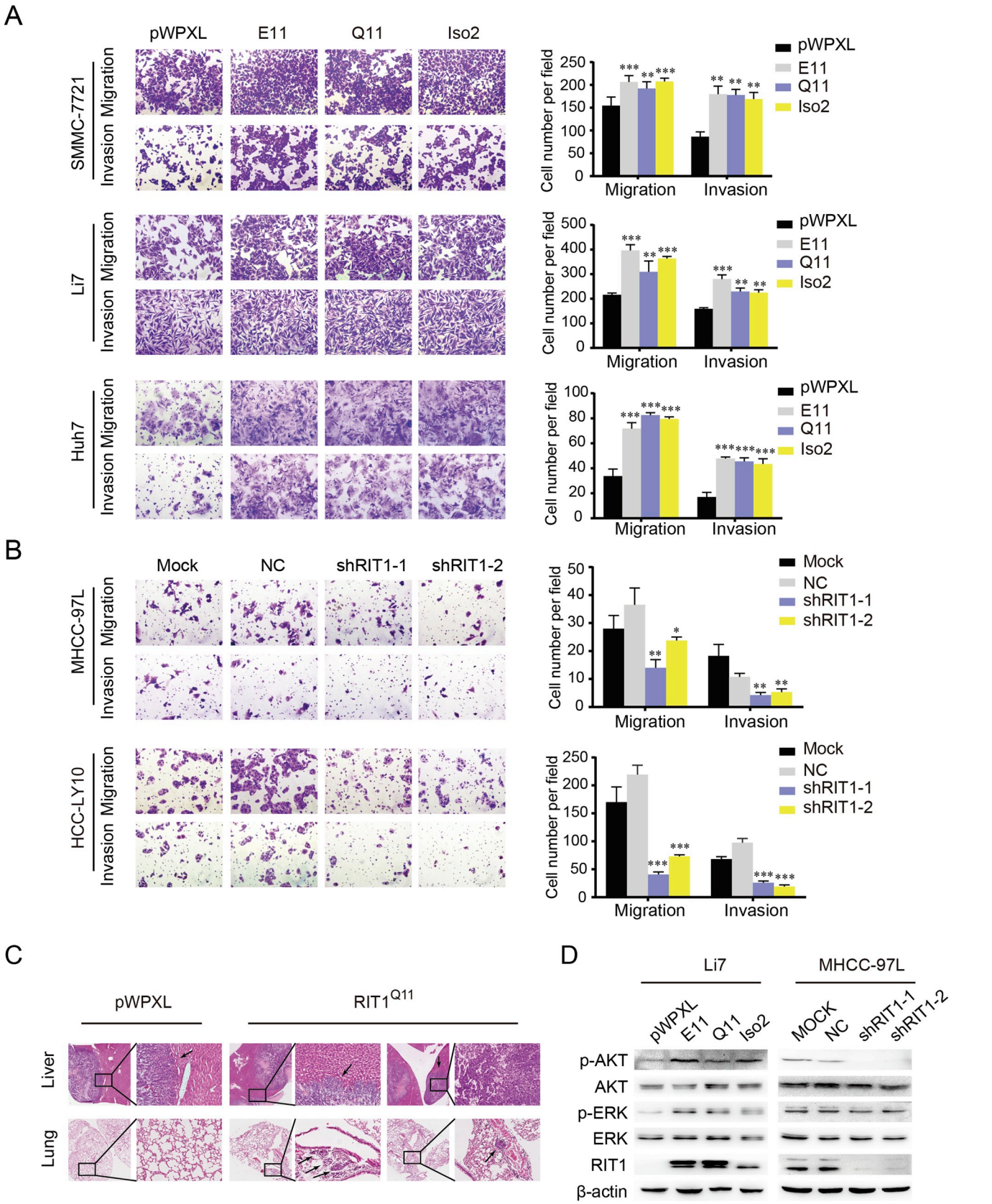

Fig. 3. RIT1 enhances HCC cell mobility *in vitro* and metastasis *in vivo*. (A) The invasion and migration abilities of SMMC-7721, Li7 and Huh7 cells stably overexpressing RIT1 were confirmed by transwell assays. (B) The invasion and migration abilities of MHCC-97L and HCC-LY10 cells with knockdown of RIT1 were confirmed by transwell assays. (C) Representative images of liver with tumors (arrows mark the tumor fronts and liver metastasis) and lung metastatic nodules (arrows) derived from SMMC-7721 cells stably overexpressing RIT1 ^{Q11} or pWPXL (control) are presented (original magnification: left × 40, right × 400). (D) Western blotting analysis of the expressions of phospho-AKT, AKT, phospho-ERK, ERK and RIT1 in RIT1-overexpressing Li7 cells and MHCC-97L cells with knockdown of RIT1. β-actin was used as a loading control. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

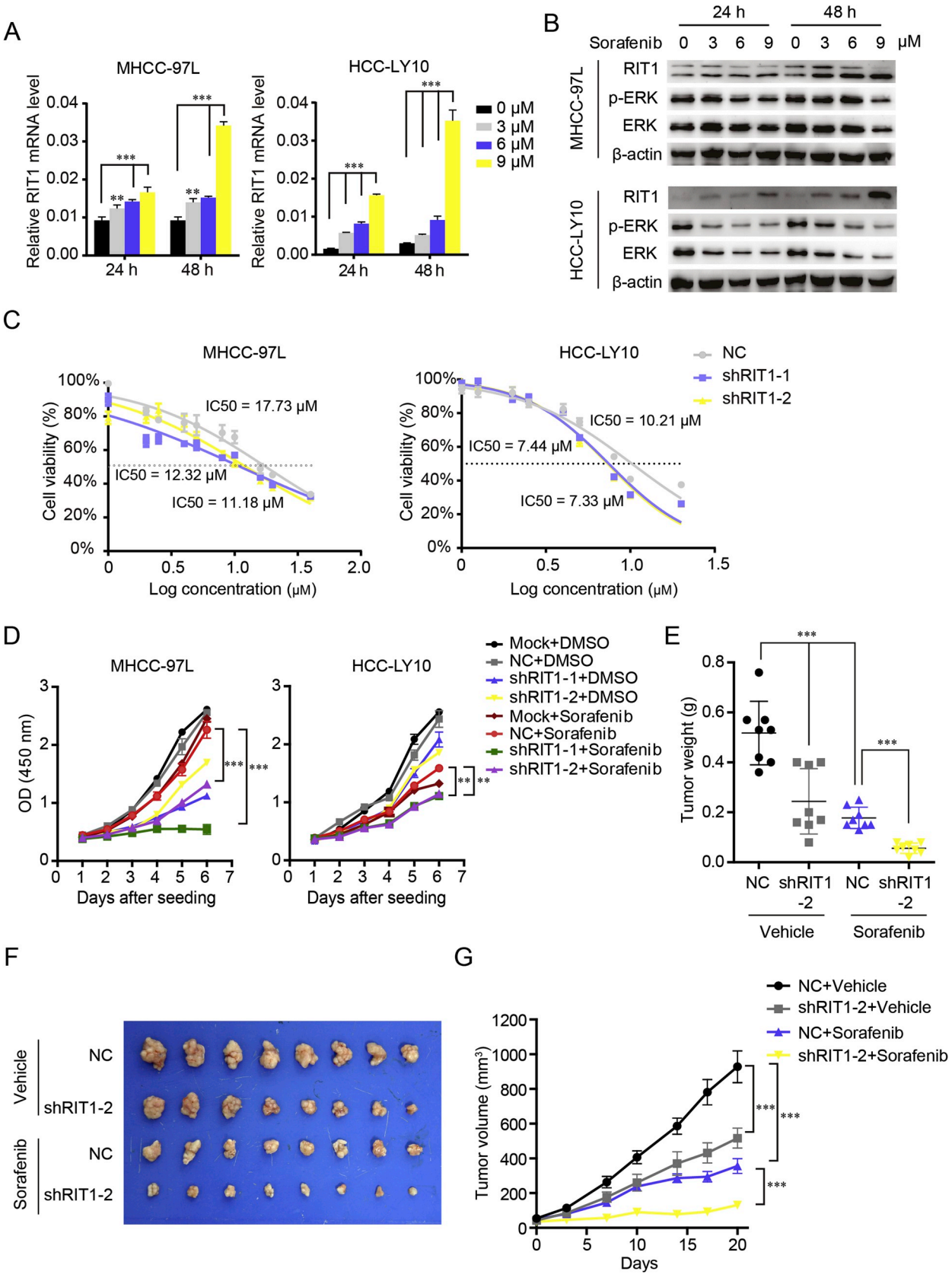

Fig. 4. Knockdown of RIT1 confers sensitivity to sorafenib in HCC cell lines. (A) qPCR and western blotting analysis (B) of MHCC-97L and HCC-LY10 cells showed RIT1 expressions were upregulated after exposure to 3 μM, 6 μM and 9 μM sorafenib for 24 h and 48 h. (C) The effects of RIT1 knockdown on IC50s of sorafenib in MHCC-97L and HCC-LY10 cells were analyzed. (D) CCK8 assays showed synergistic response to 3 μM sorafenib combined with RIT1 knockdown. (E–F) HCC-LY10 cells stably expressing NC or shRIT1 were injected subcutaneously into the flanks of nude mice administrated without or with Sorafenib. xenografts were weighted (E) and representative images of dissected tumors at the end of the experiment were shown (F). (G) tumor growth curves of mice during Sorafenib treatment were analyzed (data are shown as mean ± SEM). $^{**}P < 0.01$, $^{***}P < 0.001$.

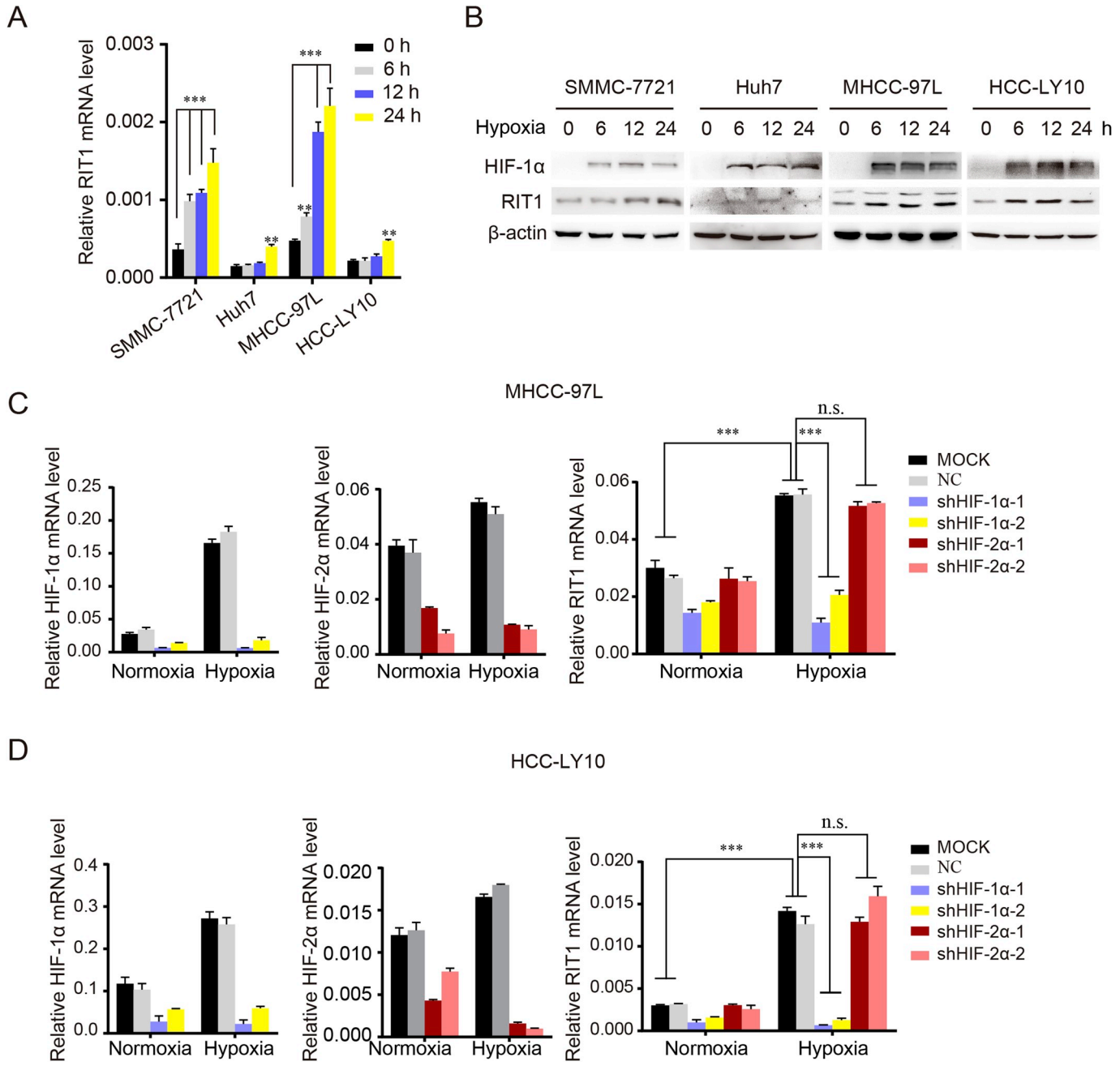

Fig. 5. HIF-1α mediates hypoxia-induced RIT1 overexpression in HCC cells. (A) qPCR and western blotting analysis (B) of SMMC-7721, Huh7, MHCC-97L and HCC-LY10 cells showed RIT1 was induced by hypoxia. (C) qPCR analysis of HIF-1α, HIF-2α and RIT1 in MHCC-97L cells with knockdown of HIF-1α or HIF-2α under normoxia and hypoxia. (D) qPCR analysis of HIF-1α, HIF-2α and RIT1 in HCC-LY10 cells with knockdown of HIF-1α or HIF-2α under normoxia and hypoxia. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

overexpression in HCC. Base on the results that only 11.44% HCC cases existed RIT1 amplification, we further investigated the regulation mechanism of RIT1 in HCC to clarify why RIT1 is overexpressed in HCC tissues. Although HCC is a highly vascularized tumor, neoplastic vessels are functionally abnormal and areas of hypoxia are common [28]. Hypoxia further triggers a series of molecular events in HCC and stimulates angiogenesis, survival, immune evasion, invasion and metastasis [28]. We showed for the first time that RIT1 was significantly upregulated when HCC cells were exposed to hypoxia. Cancer cells adapt to hypoxia by hypoxia-inducible factors (HIFs). HIFs, which mediate expression of genes that are involved in every step of HCC metastasis including epithelial-mesenchymal transition, invasion of the extracellular matrix, intravasation, extravasation, and secondary growth of the metastases, are heterodimers that consist of the constitutively expressed HIF-1β subunit and the O_2-sensitive HIF-1/2α subunit [27]. Our results showed that the binding of HIF-1α to the RIT1 promoter was a key determinant in its expression. It is reported that HIF-1α is highly expressed in HCC tissues and correlates with lymph node metastasis and decreased survival rate [29,30]. More importantly, we found that knockdown of RIT1 repressed hypoxia-induced migration and invasion and RIT1 expression was positively related with HIF-1α in HCC tissues. These data suggest that RIT1 is a functional downstream target of HIF-1α and HIF-1α plays important roles in HCC metastasis at least partly through regulation of RIT1 transcription.

Sorafenib is the standard of care for the treatment of advanced HCC [31], but it is associated with serious adverse side effects and drug

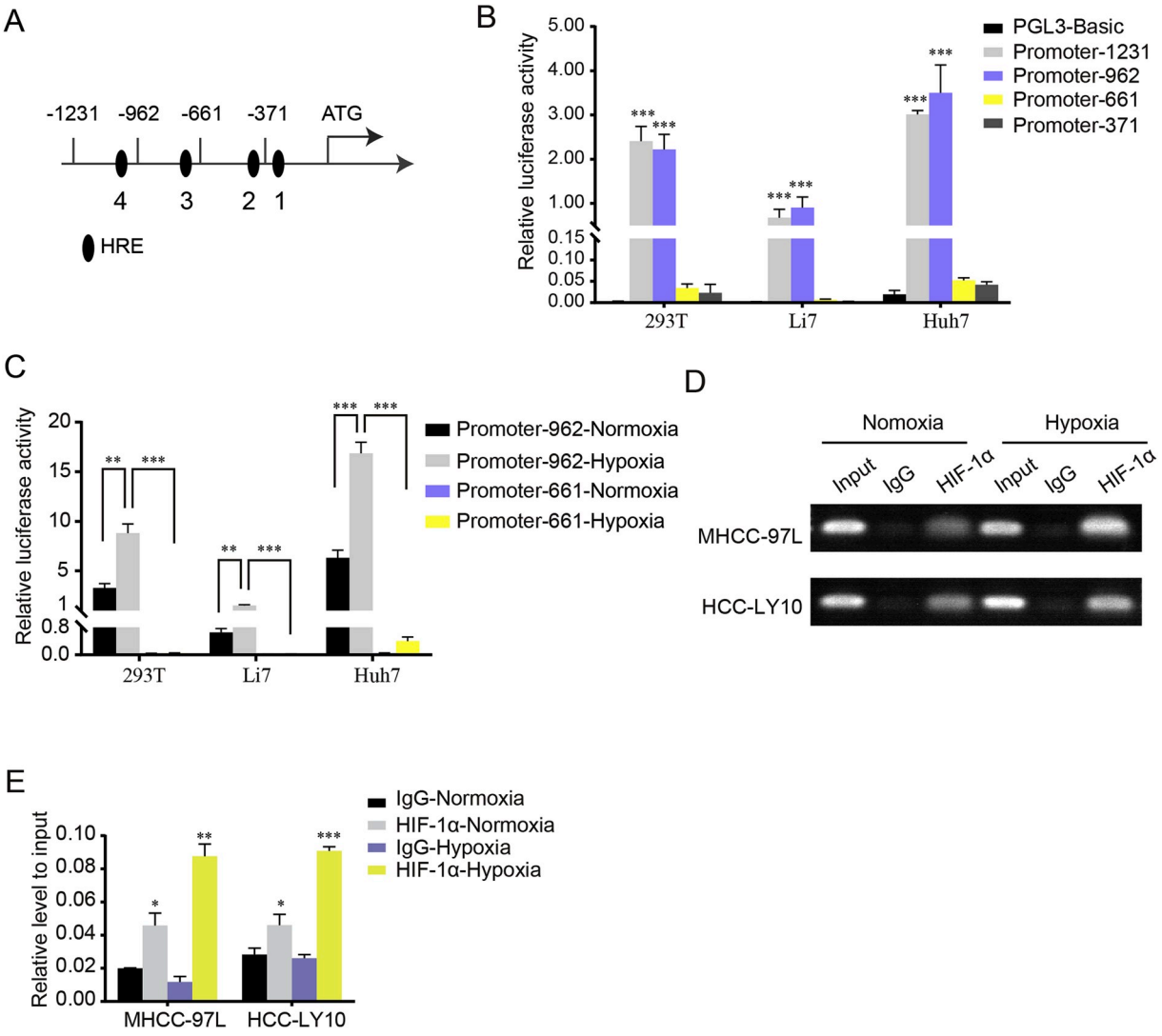

Fig. 6. RIT1 is a direct target of HIF-1α. (A) Four potential HREs in the RIT1 promoter identified with the JASPAR database (http://jaspar.genereg.net/) [35]. (B) Relative activities of the RIT1 promoter and deletion mutants in HEK 293T, Li7 and Huh7 cells were detected by dual luciferase reporter assays. (C) Relative activities of the RIT1 promoter and the deletion mutant promoter in HEK 293T, Li7 and Huh7 cells under normoxia and hypoxia. (D–E) Assessment of HIF-1α binding to the RIT1 sequence was performed by ChIP assays using an antibody against HIF-1α and a negative control (IgG) in MHCC-97L and HCC-LY10 cells under normoxia and hypoxia. Agarose gel electrophoresis (D) and qPCR (E) were used to analyze the crosslinking status. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

resistance often develops [32]. Several factors including EGFR, HER3, IGF/FGF signaling, MEK signaling and HIF-1α limit the efficiency of sorafenib [24,31–34]. We found MHCC-97L and HCC-LY10 cells were insensitive (IC50 > 10 μM) to sorafenib, which is consistent with previous report [24]. Sorafenib treatment increased RIT1 levels and knockdown of RIT1 in HCC cells displayed increased sensitivity to sorafenib. RIT1 shares effector molecules with Ras, such as Raf1 and the p110 catalytic subunit of PI3K [5]. In the present study, RIT1-induced ERK and AKT activation has also been found in HCC cells stably overexpressing RIT1 and knockdown of RIT1 can repress ERK and AKT activity. ERK2 inhibition is identified as an enhancer of the response to sorafenib in HCC [24]. These data indicate that RIT1 might affect sorafenib sensitivity via MAPK signaling, which needs further investigation and might be useful for overcoming resistance to sorafenib in HCC.

In conclusion, hypoxia induced RIT1 can significantly promote HCC cell growth, metastasis and sorafenib resistance. Furthermore, RIT1 is directly transcriptional regulated by HIF-1α and responsible for hypoxia induced migration and invasion. Our data reveal that HIF-1α/RIT1 axis plays an important role in HCC progression and sorafenib resistance and provide valuable information for HCC prognosis and treatment.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgements

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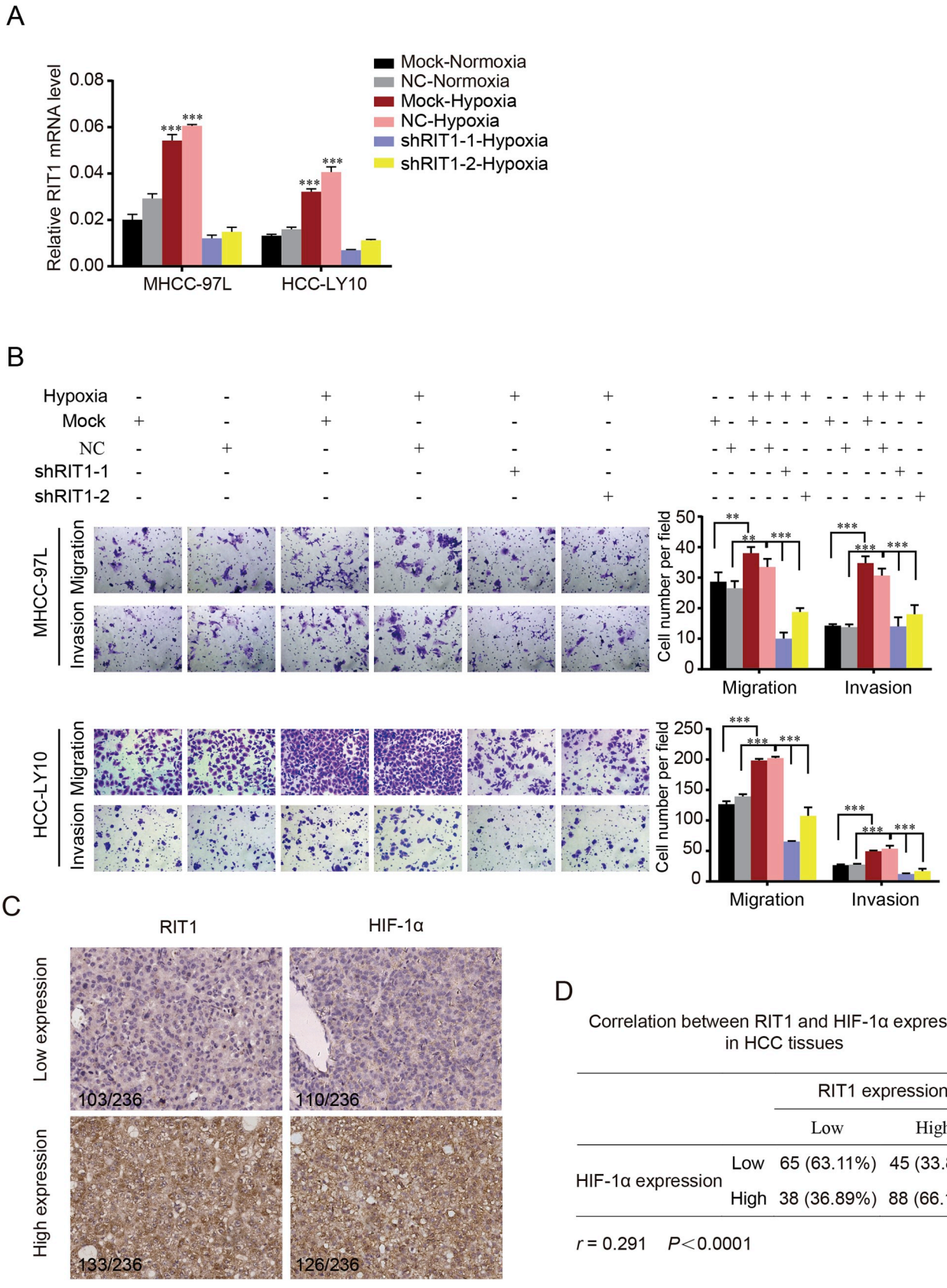

Fig. 7. Knockdown of RIT1 attenuates the invasion and migration induced by hypoxia and RIT1 expression is positively correlated with HIF-1α in HCC tissues. (A) qPCR analysis of RIT1 in MHCC-97L and HCC-LY10 cells with knockdown of RIT1 under hypoxia for 24 h. (B) Effects of RIT1 knockdown on hypoxia-induced invasion and migration were performed using transwell migration and invasion assays. (C) Immunohistochemical staining of RIT1 and HIF-1α in HCC tissues (original magnification: × 400). (D) Correlation between RIT1 and HIF-1α expression in HCC tissues. $^{**}P < 0.01$, $^{***}P < 0.001$.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.06.016.

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