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Original Paper

Hypoxia Accelerates Aggressiveness of Hepatocellular Carcinoma Cells Involving Oxidative Stress, Epithelial-Mesenchymal Transition and Non-Canonical Hedgehog Signaling

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Key Words
Hypoxia • EMT • Hedgehog • HCC • Invasion

Abstract
Background/Aims: Hypoxic microenvironment, a common feature of hepatocellular carcinoma (HCC), can induce HIF-1α expression and promote the epithelial-mesenchymal transition (EMT) and invasion of cancer cells. However, the underlying molecular mechanisms have not fully elucidated. ***Methods:*** HCC cells were cultured under controlled hypoxia conditions or normoxic conditions. Transwell assays were used to examine the migration and invasion capacity. HIF-1α siRNA, cyclopamine (a SMO antagonist) and GLI1 siRNA were used to inhibit HIF-1α transcription or Hh signaling activation. ***Results:*** In present study, we first observed a strongly positive correlation between HIF-1α and GLI1 expression in HCC tissues. Then, we showed that hypoxia significantly promoted EMT process and invasion of HCC cells, associated with activating the non-canonical Hh pathway without affecting SHH and PTCH1 expression. HIF-1α knockdown mitigated hypoxia-induced SMO and GLI1 expression, EMT invasion of HCC cells. Moreover, the SMO inhibitor or GLI1 siRNA also reversed the hypoxia-driven EMT and invasion of HCC cells under hypoxia condition. Here, we show that non-canonical Hh signaling is required as an important role to switch on hypoxia-induced EMT and invasion in HCC cells. In addition, we found that hypoxia increased ROS production and that ROS inhibitors (NAC) blocked GLI1-dependent EMT process and invasion under hypoxic conditions. To determine a major route of ROS production, we tested whether nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 4 (NOX4) is involved in hypoxia-induced ROS production. NOX4 expression was found to be increased at both mRNA and protein levels in hypoxic HCC cells. Furthermore, siRNA-mediated knockdown of NOX4 expression abolished

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hypoxia induced ROS generation and GLI1-dependent activation and invasion of HCC cells. ***Conclusion:*** Our findings indicate that hypoxia triggers ROS-mediated GLI1-dependent EMT progress and invasion of HCC cells through induction of NOX4 expression. Thus, hypoxia-driven ROS mediated non-canonical Hh signaling may play an important role in the initiation of EMT and provides a potential marker for cancer prevention and treatment.

Introduction

Hepatocellular carcinoma (HCC), one of the most usual malignant tumors, was the third dominant reason of cancer-related deaths worldwide [1]. Although effective diagnosis and treatment during past decades, the post-operation outcome of HCC was still dismal due to the high tumor recurrence or distant metastasis rate [2]. Nevertheless, the detailed mechanisms for the HCC metastasis has not been elucidated [3]. Thus, it is emergency to discover new comprehensive treatment for HCC.

Hypoxia is a prevalent tumor microenvironment on account of redundant oxygen consumption in rapidly growing tumor [4]. Accumulating evidence indicates that tumor hypoxia strongly facilitates tumor invasiveness, angiogenesis, phenotype conversion and distant metastasis [5, 6].The insufficient oxygen in hypoxia microenvironment limits tumor cell division, while selects more malignant cells to change into metastasis type and treatment resistance [7]. The functional effects of oxygen deprivation are principally mediated by the activation of hypoxia-inducible transcription factor-1 (HIF-1) who contains a constitutively expressed non-oxygen-sensitive β-subunit and an oxygen-dependent α-subunit [8]. HIF-1α enhances cancerous adaption to hypoxia stress by regulating target genes, including VEGF, CXCR4, which is ectopic-expression in diverse solid cancers, including HCC [9]. In hypoxia environment, the intracellular reactive oxygen species (ROS) levels paradoxically increase through oxygen metabolism which occur under cellular oxidative stress conditions and exert critical function in the homeostasis via redox pathways [10-12]. Increased amounts of ROS are generated under hypoxic phase from sources such as NADPH oxidase (NOX)[13, 14], or the damage of the protective Nrf2 signaling against oxidative stress [15]. Increasing studies suggest that ROS serve as a vital signaling molecule in cancer cell proliferation, migration, metastasis and cancer EMT phenotype. However, the ROS-related signaling involved in the HCC under hypoxia conditions has not been well investigated so far.

EMT has been recognized as a key process of the invasion and metastasis in multiple cancers, including HCC [16-18]. EMT is marked of losing epithelial adhesion and cytoskeletal markers [19], such as E-cadherin, meanwhile the obtaining of migratory mesenchymal symbols, such as N-cadherin and Vimentin. Several reports demonstrated that EMT is modulated via many transcriptional factors, including Snail, Twist1. These processes increase aggressiveness and promote the metastatic potential of HCC [16, 20]. Therefore, identifying specific therapeutic targets in EMT and elucidating the underlying mechanisms may ultimately contribute to suppress the malignancy of HCC.

The Hedgehog (Hh) pathway is involved in development, homeostasis and stem cell maintenance [21]. Its aberrant activation can promote the EMT process, invasion and metastasis of HCC [22-24]. The Hh pathway is starting with the interacting of SHH ligand to transmembrane receptor, patched1 (PTCH1), which relieves its suppressive function on membrane-spanning protein SMO. Once activated, this de-repression SMO eventually leads to a complex series of intracellular activity of the glioma-associated oncogenes (GLI), which regulates target genes including PTCH1 and GLI1[25, 26]. Thus, SMO and GLI1 expression was identified as the molecular signs of the Hh signaling activation. Accumulating reports have shown that the Hh pathway has a vital character in different tumors, including HCC [27-29]. Our previous studies reveal that the overexpressed of GLI1 in HCC are associated with the invasiveness of HCC, and its up-regulation predicts poor prognosis and represent the progressive stages of HCC [30]. GLI1 is a key transcription factor of Hh signaling. GLI1 knockdown by RNA interference restrains cell invasion and EMT in HCC [31]. Moreover,

some studies recently showed that numerous molecular signaling pathways, containing Hh pathway [32], could be activated under hypoxia conditions [33, 34]. However, the function of Hh pathway involved in the hypoxia-driven EMT and metastasis of HCC cells together with its relation with HIF-1α-induced ROS remain unclarified.

In the research, we concentrated on defining the specific process by which hypoxia-induced ROS stimulate EMT progress and invasion capacity under hypoxia through Hh pathway in HCC cell lines. We show that hypoxia-driven EMT is dependent upon the key Hh pathway transcription factor GLI1 overexpression, that this increased expression is due to the activation of hypoxia-driven ROS. In addition, we show that hypoxia induced ROS activities through the excessive enhancement of NOX4-mediated generation. In conclusion, we demonstrated that non-classical Hh pathway is responsible for HIF-1α/NOX4/ROS under hypoxia condition to modulate the EMT process and invasion capacity in HCC cells.

Materials and Methods

Patients' tissues and cell culture

Patients' tissues and paired adjacent non-tumor tissues were obtained from 126 patients in our department between January, 2006 and December, 2009 after the informed consent were obtained from all patients. They didn't receive any therapy before surgery. Xi'an Jiaotong University Ethics Committee approved the research on the basis of Declaration of Helsinki.

The normal immortalized human hepatocyte LO2 and a panel of HCC cells (MHCC-97H, Hep3B, Huh7, MHCC-97L and HCCLM3) (Chinese Academy of Sciences, Shanghai, China) were maintained in DMEM (Invitrogen, Carlsbad, USA) containing 10% FBS (Gibco, GrandIsland, USA) at the normoxic incubator in 37˚C with 5% CO2. To explore the function of hypoxia, Hep3B and MHCC-97L cells were cultured to 60-70% confluence in 3% O_2 hypoxic condition for 48h. SMO inhibitor cyclopamine (10 μM, Selleck Chemicals, Houston, TX, USA) was dealt with the manufacturer's instructions.

RNA extraction and qRT-PCR

QRT-PCR was conducted as reported previously [35, 36]. All RNA was extracted based on the protocol of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). qPCR primer against HIF-1α (HQP008831), SHH (HQP017098), PTCH1 (HQP015530), SMO (HQP017563), GAPDH (HQP006940), GLI1 (HQP007701), VEGF (HQP018475) and NOX4 (HQP012143) were ordered from Genecopoeia (Guangzhou, China).

Immunohistochemical staining (IHC)

The sections were dewaxed, dehydrated, and rehydrated. HIF-1α, GLI1 (1:100, Cell Signaling, Danvers, MA, USA) were added to the sections and incubating at 4°C overnight. Then applying the biotinylated secondary antibodies (Goldenbridge, Zhongshan,China) according to SP-IHC assays.. Specific experiment was conducted similar to previously reported [35, 36].

Immunofluorescence (IF)

We used 4% paraformaldehyde to fix transfected cells and used 0.3% Triton X-100 to permeabilize. The primary antibody GLI1, NOX4 (1:200, CST, Danvers, USA) was used. Then the Alexa Fluor-conjugated secondary antibody was performed the next experiment. Lastly, the images were taken by Microscope (Zeiss, Germany).

Western blot analysis

We separated proteins by SDS–PAGE and transferred proteins to PVDF membranes. Detailed experiment was performed similar to previously reported [35, 36].

Measurement of ROS

The ROS levelwas analyzed with use of an ROS assay kit (GMS10016.2, Gemmed Scientifics, USA) based on protocol. Cells were incubated with DCFH-DA probe at 1:1000 dilutions in the condition and maintained at 37°C for 30 min, following serum-free IMDM medium washing three times and visualized

under fluorescent microscopy and measured by use of a Micro Fluorescence Reader with excitation at 490 nm (BIO-TEK Instruments).

RNA interference transfection

The HIF-1α, GLI1, NOX4 and a negative control siRNA were synthesized by GenePharm (Shanghai, China). Hep3B and MHCC-97L cells (2×10^5 per well) were transfected in a concentration of 100 nM siRNA.

Transwell migration and invasion assay

The upper chambers of transwell inserts (8μM pore-sized,Nalge Nunc, NY, USA) were coated with matrigel (1:8,BD, NJ, USA) for invasion assay, while migration assay without matrigel. The upper chamber cells were 2.5×10^4 in DMEM, and the bottom was 10% FBS DMEM. After 24 h, the cells were fixed using 4% paraformaldehyde, permeabilized with methanol, then stained with crystal violet.

Statistical analysis

Results are managed as the mean ± SD and analyzed bySPSS software, 16.0 (SPSS, Chicago, USA). The statistical approaches mainly included a two-tailed Student's t test, a Kaplan–Meier plot, Pearson chi-squared testand so on. Difference with $P < 0.05$ was regard to be significant. Graphs were mainly made by GraphPad Prism 6 (GraphPad, San Diego, USA).

Results

GLI1 was up-regulated in HCC tissues and cell lines and positively correlated with HIF-1α expression

The positive relationship between HIF-1α and GLI1 was reported previously in pancreatic and breast cancer cell lines [37, 38]. To investigate the potential function of Hh signaling and the role of HIF-1α in the initiation of EMT course of HCC. First, we detected HIF-1α and GLI1 levels in 126 HCC tissues by IHC. Both HIF-1α and GLI1 in cancer tissues were obviously up-regulated compared with paired non-cancerous tissues (P<0.05, Fig. 1A,

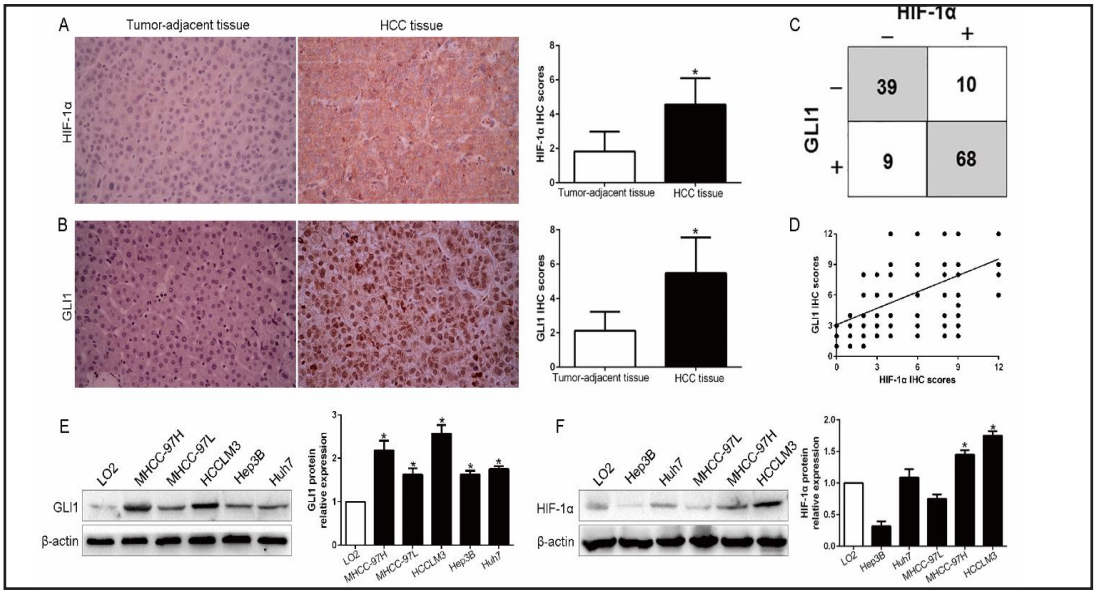

Fig. 1. GLI1 was overexpressed in HCC tissues and a panel of cell lines, meanwhile its expression was positively associated with HIF-1α expression. (A) The representative HIF-1α expression in HCC tissues and non-tumor tissues. (B) The representative GLI1 expression in HCC tissues and non-tumor tissues. (C) An obviously positive association between HIF-1α and GLI1 expression was discovered in HCC tissues. $P < 0.05$ by two-tailed chi-square test. (D) The Spearman rank analysis revealed the positive association between HIF-1α and GLI1 expression in HCC samples (n=126, r=0.5360, $P < 0.0001$). Western blot results of GLI1 (E) and HIF-1α (F) protein expression in a panel of cell lines. *P<0.05.

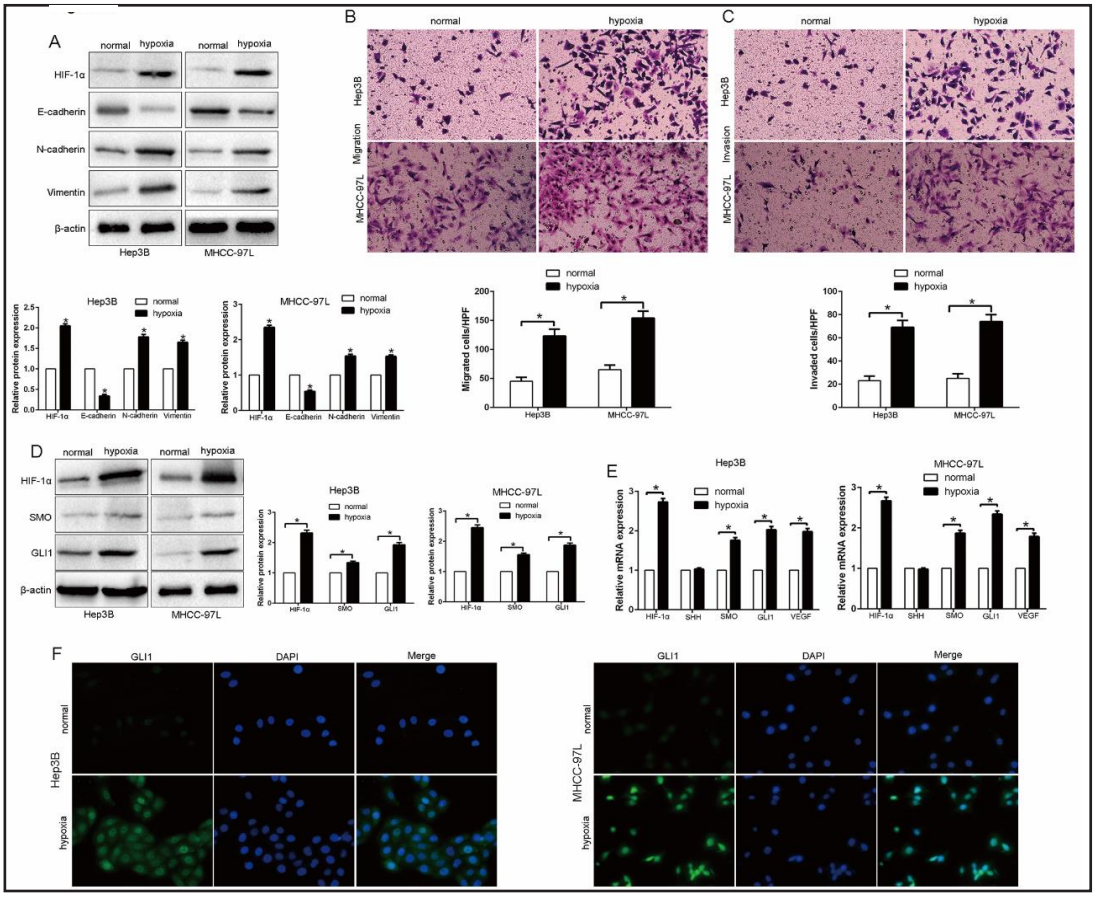

Fig. 2. Hypoxia induces HIF-1α and facilitates Hh signaling to induce an EMT process and promote invasive capacity in MHCC-97L and Hep3B cells. (A) Western blot analysis of E-cadherin, N-cadherin and Vimentin of Hep3B and MHCC-97L cells cultured in normoxic or hypoxic conditions. Cell migration (B) and invasion (C) as measured by Transwell assays were increased in hypoxic condition compared to normoxic condition. (×100). Below, quantification of migration and invasion. (D) Western blot analysis of related Hh signaling protein. (E)The HIF-1α, SHH, SMO, GLI1 and VEGF mRNA were detected using qRT-PCR. (F) Immunofluorescence staining of GLI1 in Hep3B and MHCC-97L cells cultured in normoxic or hypoxic condition. HPF: high power field. *P<0.05 by t test.

B). In these samples, GLI1 expression was stained in 87.2% (68/78) of the HCC tissues with positive HIF-1α expression, similarly, 81.3% (39/48) of HCC specimens with negative HIF-1α expression showed a homologous GLI1 expression (P<0.05, Fig. 1C). In addition, the data revealed that HIF-1α was positively associated with GLI1 expression (r=0.5360, P<0.001; Fig. 1D). This information revealed that HIF-1α probably exert its biological promotive function through GLI1 expression. Next, we explored the expression of HIF-1α and GLI1 in cell lines. We found GLI1 were up-regulated in a panel of HCC cell lines than normal hepatocyte LO2 (P<0.05, Fig. 1E). Moreover, the HIF-1α was also measured but different between HCC cell lines (P<0.05, Fig. 1F).

Hypoxia accumulates HIF-1α and facilitates Hh signaling to induce an EMT process and promote invasive capacity in MHCC-97L and Hep3B cells

To confirm whether hypoxia could induce EMT, we measured the markers of EMT expression using Western blot. E-cadherin was reduced, whereas that of N-cadherin and Vimentin was increased in hypoxia cells (Fig.2A). Moreover, the migrated and invaded cells from hypoxia condition were markedly increased than normoxia (P<0.05, Fig. 2B,

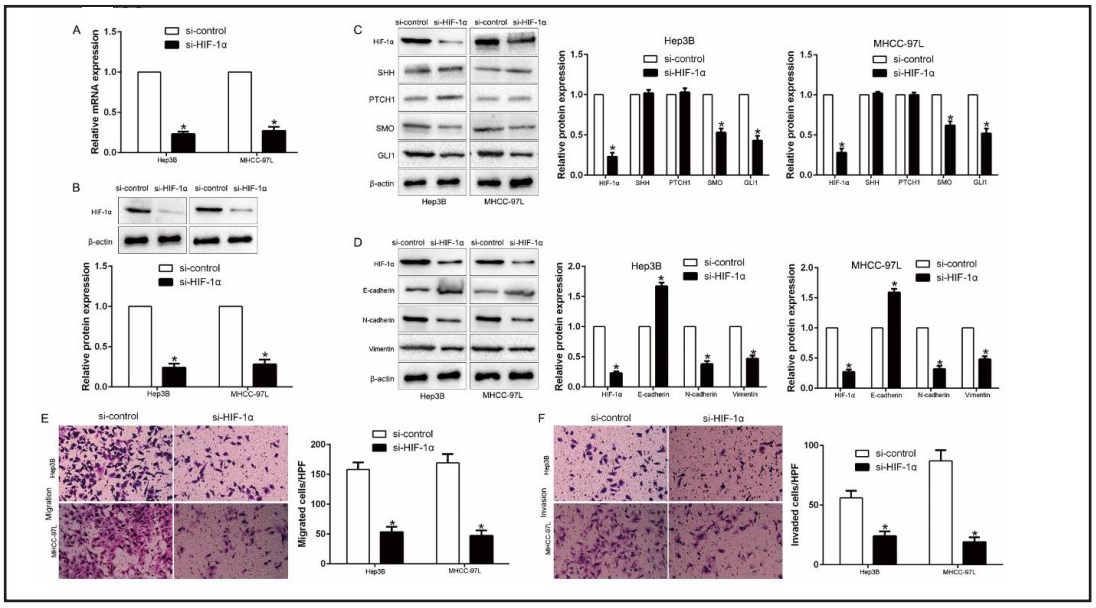

Fig. 3. HIF-1α is a necessary mediator of hypoxia-induced changes of Hh pathway, EMT phenotype and invasion capacity in HCC cells. (A) HIF-1α mRNA was measured after transfecting with siRNA in both cells. (B) HIF-1α expression in siRNA-mediated knockdown in Hep3B and MHCC-97L cells analyzing by Western blot. (C) The expression of Hh signaling protein level were determined by Western blot after transfection. (D) The function of HIF-1α knockdown on EMT. The impaction on cell migration (E) and invasion (F) after HIF-1α knockdown cultured in hypoxic conditions. *P<0.05 by t test.

C). Previous reports reported that the promotive function by the hypoxia environment is dominantly mediated by HIF-1α, which can activate downstream signaling pathway to affect the proliferation, invasion and metastasis of cancer cells [39]. Evidences has confirmed that hypoxia promoted the invasive ability and EMT of pancreatic cancer through Hh pathway [40]. To clarify the mechanisms of the hypoxia-induced HCC metastasis, MHCC-97L and Hep3B cells were incubated in different oxygen condition and the Hh expression were checked. The HIF-1α, SMO and GLI1 expression were obviously up-regulated in hypoxic condition of both cell lines than normoxic condition (P<0.05, Fig. 2D). Moreover, the relative amount of HIF-1α, SMO and GLI1 mRNA were also markedly elevated, together with its target gene VEGF, however, SHH mRNA remained unchanged compared to normoxic condition (Fig.2E). In addition, immunofluorescence for GLI1 indicated that exposure to hypoxic induced GLI1 nuclear translocation (Fig.2F, Fig.7A). These data indicated that hypoxia promoted Hh signaling activation in both HCC cell lines.

HIF-1α is a necessary mediator of hypoxia-induced changes of Hh pathway, EMT phenotype and aggressive capacity in HCC

To confirm the critical function of HIF-1α on hypoxia-induced effects, we transfected a specific HIF-1α siRNA to knockdown the expression levels of both mRNA and protein in Hep3B and MHCC-97L cells in hypoxia condition (P<0.05, Fig. 3A, B). As expected, downregulated HIF-1α contributed to a significant reduction of SMO and GLI expression in both cells, however, no effect was observed in SHH and PTCH1 expression (P<0.05, Fig. 3C). Moreover, HIF-1α knockdown obviously inhibited N-cadherin and Vimentin, whereas promoted the E-cadherin level (P<0.05, Fig. 3D). Furthermore, we also explored biological function of HIF-1α in the hypoxia condition. We found HIF-1α silenced resulted in a significantly decreased migration and invasion (P<0.05, Fig. 3E, F). These data demonstrated that the activated Hh signaling, EMT phenotype progress and the enhanced invasive ability under hypoxia condition was mediated of HIF-1α.

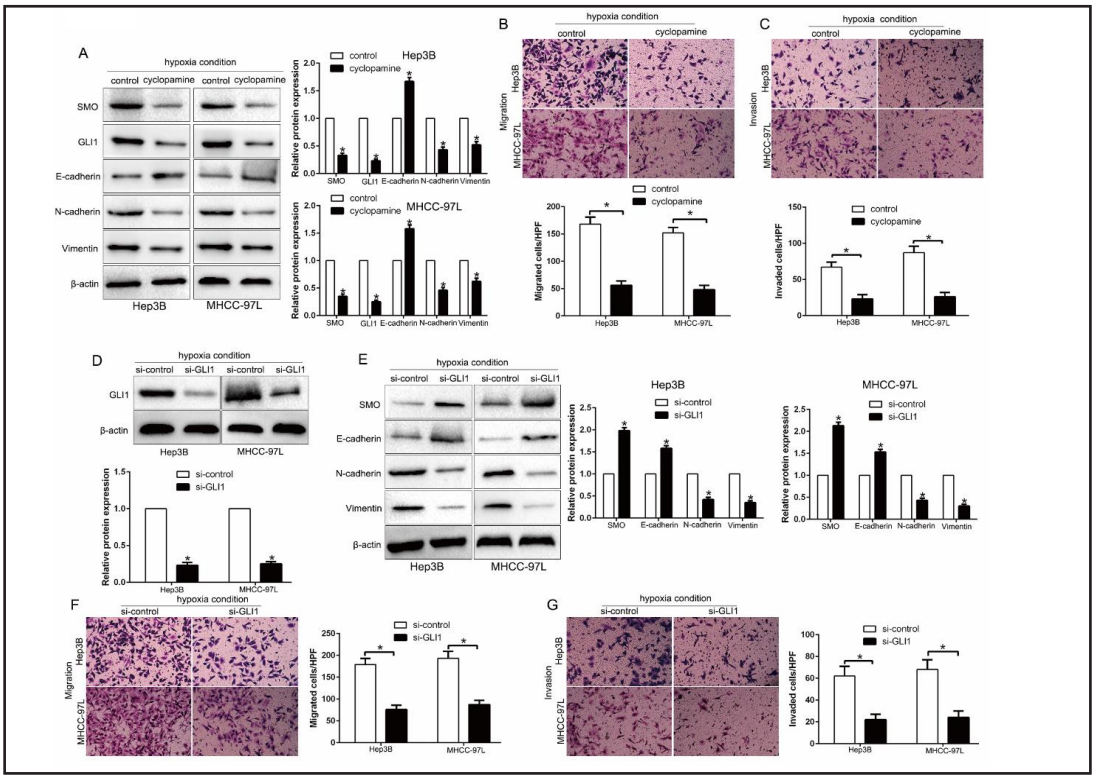

Fig. 4. Alteration of Hh signaling mitigates hypoxia-enhanced EMT phenotype and invasion capacity in HCC cells. (A) Western blot determined SMO, GLI1 and EMT markers expression after treating cyclopamine. The Smo inhibitor, cyclopamine, affects cell migration (B) and invasion (C) under hypoxia conditions. (D) Western blot measuredGLI1 expression after siRNA transfecting into Hep3B and MHCC-97L cells. (E) Western blot detected the SMO and EMT expression after GLI1 knockdown. GLI1 knockdown affects cell migration (F) and invasion (G) cultured in hypoxic conditions. *P<0.05 by t test.

Alteration of Hh signaling abolished hypoxia-driven EMT progress and aggressive capacity in HCC cells

The obtained results suggest that hypoxia enhanced HCC EMT and invasion by affecting the expression of SMO and GLI1 expression, but not SHH expression. To confirm the importance of SMO and GLI1 in modulating hypoxia-induced EMT process and invasion, we used the SMO antagonist (cyclopamine) and specific GLI1 siRNA to suppress the Hh signaling who was activated in hypoxia condition. that the data revealed that cyclopamine obviously inhibited the SMO and GLI1 expression, reversed the EMT process (P<0.05, Fig. 4A) and suppressed the migration and invasion (P<0.05, Fig. 4B, C) under hypoxia condition. In addition, we also used specific GLI1 siRNA to knockdown GLI1 in HCC cells (P<0.05, Fig. 4D). As expected, E-cadherin was significantly increased, while N-cadherin and Vimentin were obviously down-regulated. Interestingly, the SMO was even increased in hypoxia condition (P<0.05, Fig. 4E). Moreover, down-regulated of GLI1 markedly abolished HCC migration and invasion (P<0.05, Fig. 4F, G) induced by hypoxia condition. In conclusion, these data revealed that hypoxia-driven EMT progress and aggressive capacity of HCC cells were mediated by GLI1.

Hypoxia-induced intracellular ROS production is crucial for the activation of GLI1-dependent signaling, EMT progress and invasion of HCC cells

Previous reports have confirmed that ROS plays a significant effect in cancer progression [41]. Moreover, Lim et al [42]. demonstrated that ROS accumulation played a vital role in the regulation of the Hh pathway, especially the GLI1, GLI2 expression. To explore whether

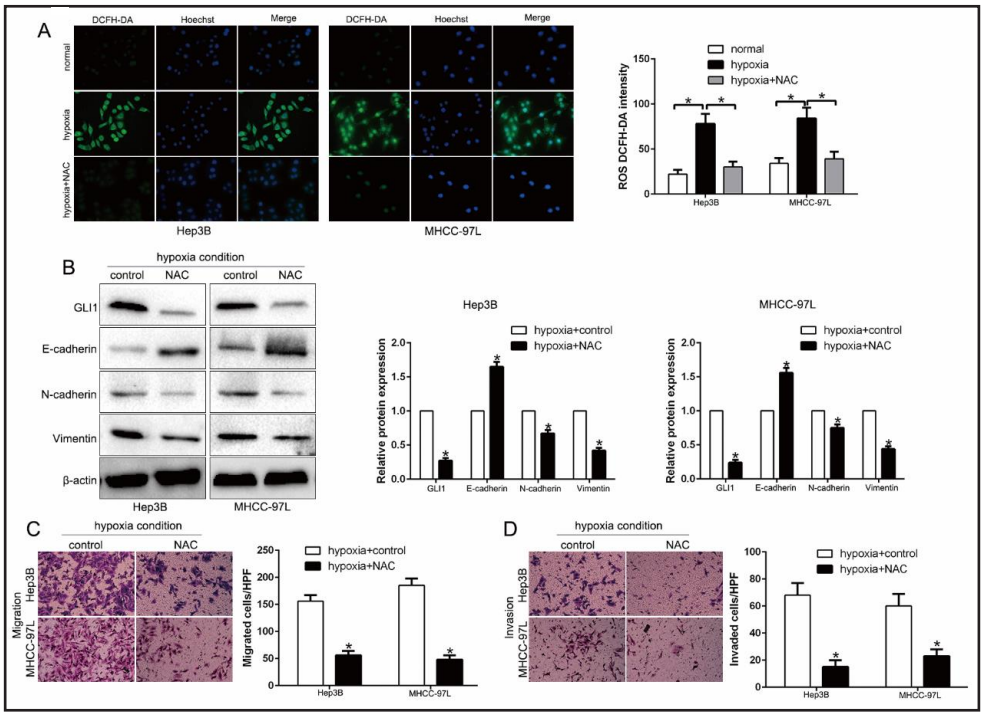

Fig. 5. Hypoxia-driven intracellular ROS accumulation plays a critical role in the activation of GLI1-dependent signaling, EMT progress and invasion of HCC cells. (A) Hep3B and MHCC-97L cells were stained with 5 μm DCFDA for 30 min after culturing in hypoxia for 48 h. *P<0.05 by Student's t test. (B) At 48 h after NAC treatment under hypoxia condition, Hep3B and MHCC-97L cells were subjected to immunoblot. The GLI1 and EMT-related molecular were determined. ROS elimination by NAC affects cell migration (C) and invasion (D) cultured in hypoxia conditions with NAC treatment.

hypoxia induced ROS production, we perform DCFDA to measure intracellular ROS. We found the intensity under hypoxia exposure was obviously elevated compared to normoxic (P<0.05, Fig. 5A). To explain the relationship between ROS production and GLI1-dependent EMT progress and invasion, we decreased the ROS level in hypoxia by the ROS inhibitor N-acetylcysteine (NAC) (P<0.05, Fig. 5A). Remarkably, NAC treatment in hypoxia condition obviously inhibited the GLI1 expression and EMT progression (P<0.05, Fig. 5B, Fig.7B). Moreover, the migrated (P<0.05, Fig. 5C) and invaded (P<0.05, Fig. 5D) cells cultured in NAC treatment were prominently decreased in hypoxia condition. These data revealed that ROS scavenging eliminated hypoxia-induced GLI1-dependent EMT progress and invasion of HCC cells.

NOX4 is crucial for hypoxia-induced ROS production to activate GLI1-dependent EMT process and invasion of HCC cells

Due to NOX produces superoxide through shifting electrons to oxygen, we believe that NOX could be initial factors of ROS production in hypoxia condition, especially NOX4. The data showed that NOX4 was up-regulated in HCC tissues compared to adjacent non-tumor tissues (P<0.05, Fig.7C). To investigate the roles of NOX4, Hep3B and MHCC-97L cells were maintained in the normoxic or hypoxic condition for 48h and the NOX4 expression were detected. The HIF-1α and NOX4 mRNA and protein (P<0.05, Fig. 6A, B) in hypoxia condition were markedly higher than normoxic. Consistently, immunofluorescence for NOX4 was similar with western blot. NOX4 was increased both in the cytoplasm and nucleus under hypoxia condition compared to normoxic (P<0.05, Fig. 6C). To evaluate the role of NOX4 further, we transfected NOX4-specific siRNA to knockdown NOX4 under hypoxia condition (P<0.05, Fig. 6D). Transfection with NOX4 siRNA dramatically downregulated ROS production and HIF-1α in HCC cells under hypoxia (P<0.05, Fig. 6E, Fig.7D), demonstrating the critical role of NOX4 in the ROS accumulation under hypoxia condition. We confirmed that HIF-1a

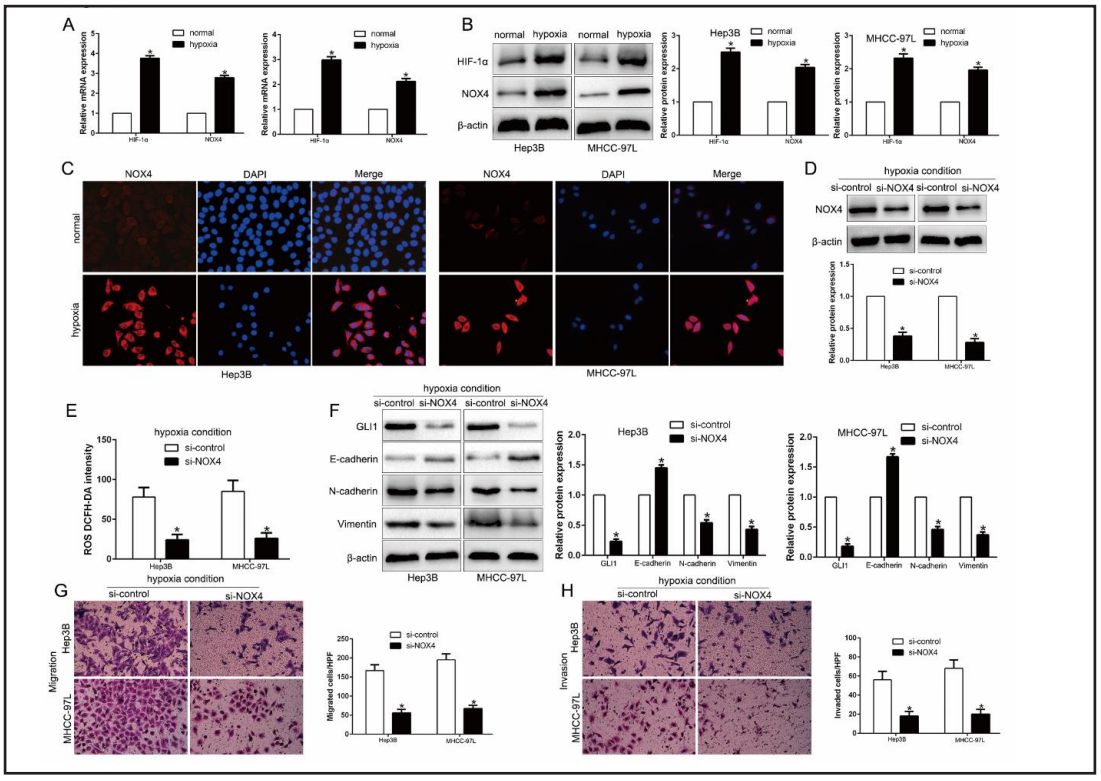

Fig. 6. NOX4 is crucial for hypoxia-induced ROS production to activate GLI1-dependent EMT process and invasion of HCC cells. (A) NOX4 mRNA level were detected after incubation 48 h under hypoxia condition. (B) Western blot detection of NOX4 in Hep3B and MHCC-97L cells after incubation 48 h under hypoxia condition. (C) Immunofluorescence staining of NOX4 in Hep3B and MHCC-97L cells under hypoxic condition for 48h. (D) Western blot analysis of NOX4 in siRNA-mediated knockdown in Hep3B and MHCC-97L cells under hypoxic condition for 48h. (E) The function of NOX4 knockdown on the generation of ROS. (F) NOX4 knockdown affects GLI1 and EMT expression. NOX4 knockdown affects cell migration (G) and invasion (H) cultured in hypoxic conditions. *P<0.05 by t test.

knockdown decreased NOX4 expression in hypoxia condition (P<0.05, Fig.7E). However, we demonstrated there was no change after GLI knockdown on HIF1a and NOX4 expression in hypoxia condition (P<0.05, Fig.7F). Moreover, we found downregulated NOX4 blocked hypoxia-induced elevation of GLI1 expression and reversed the EMT progress (P<0.05, Fig. 6F). More interestingly, NOX4 knockdown significantly lead to the decreased migrated and invaded cells cultured in hypoxia condition (P<0.05, Fig. 6G, H). Collectively, these data indicated that NOX4 was a key mediator in hypoxia-driven ROS-induced GLI1-dependent EMT progress and aggressive capacity of HCC cells.

Discussion

Hypoxia environment is commonly found in solid tumors, including hepatocellular carcinoma, which resulted from imbalance of the high proliferation rate of tumor cells and abnormal vascularization [43]. Previous reports showed hypoxia play a crucial role in metabolism, stemness and EMT phenotype [6]. EMT is a pathological mechanism in cancer development during which cells lose their cell polarity, and acquire increased metastasis mesenchymal cells [16]. HIF-1α, which is produced under hypoxia, confers promotion on EMT and plays an adaptive role. Although studies have been reported that HIF-1α indirectly regulate NF-κB and Wnt/β-catenin pathway to accelerate EMT and aggressive capacity of

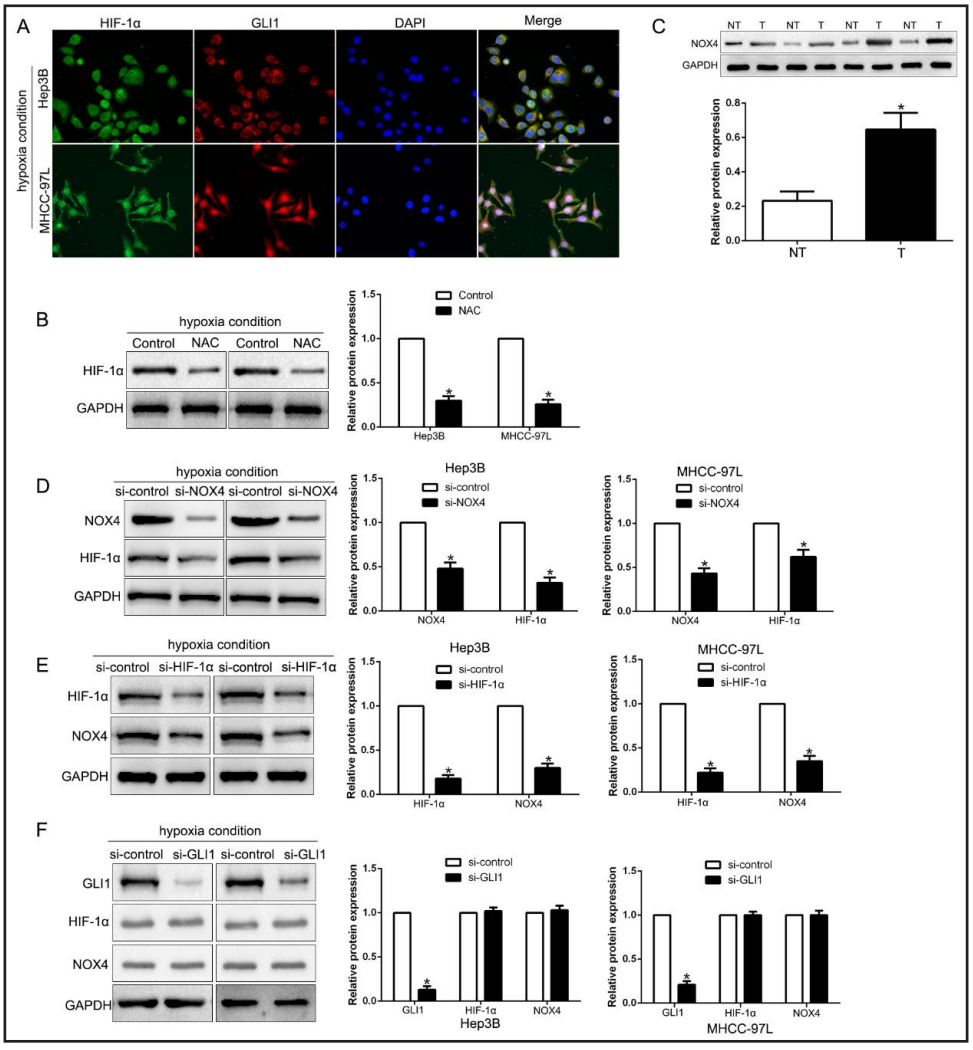

Fig. 7. (A) IF measured the co-expression of HIF-1a expression and GLI1 expression in hypoxia condition. (B) NAC inhibited HIF-1α expression in hypoxia condition. (C) NOX4 was up-regulated in HCC tissues compared to adjacent non-tumor tissues. (D) NOX4 knock-down decreased HIF-1α expression in hypoxia condition. (E) HIF-1α knockdown decreased NOX4 expression in hypoxia condition. (F) There was no change after GLI knockdown on HIF-1α and NOX4 expression in hypoxia condition.

cancer cells [44], nevertheless, the molecular mechanisms of HIF-1α mediates EMT in HCC were still unclear.

Previous study reported that hypoxia promote EMT process and facilitate the invasion of pancreatic cancer cells through a non-canonical Hh pathway [37]. To confirm that Hh pathway was involved in the hypoxia condition, we first confirm that HIF-1α and GLI1, which is a key transcriptional factor of Hh pathway, were both increased in HCC tissues and cells than non-tumor tissues and cells. Moreover, they also had a strongly positive correlation. High HIF-1α or GLI1 expression was strongly correlated with the adverse prognosis and progressive stages of HCC. Furthermore, we demonstrated that accumulated HIF-1α attributable to hypoxia promoted EMT progress and invasion of HCC cells via activation of the non-canonical Hh. We confirmed that hypoxia condition significantly promoted the SMO and GLI1 expression in both Hep3B and MHCC-97L cells, but it did not impact the SHH and PTCH1 expression. HIF-1α knockdown eliminated the hypoxia-enhanced SMO and GLI1 expression, EMT process and invasion capacity of HCC cells. According to preceding research which revealed that hypoxia induced canonical Hh pathway via HIF-1α, these data suggest that HIF-1α may be crucial for the hypoxia-induced Hh pathway to strengthen the EMT progress and aggressive capacity of HCC cells.

The Hh pathway, which was activated through secreted Hh binding to PTCH1, leading to SMO dissociating, and the GLI family nuclear translocation [26]. SMO and GLI1 have been identified as the signs of the Hh activation. To confirm the roles of different Hh signaling components levels in hypoxia, we used SMO inhibitor cyclopamine to suppress its activity or GLI1 siRNA to inhibit Hh pathway. Our results showed that blocking SMO function significantly inhibited GLI1 expression, reversed EMT progress and invasion induced by hypoxia. Furthermore, GLI1 knockdown suppressed EMT and invasion induced by hypoxia, however, decreased GLI1 could not affect the hypoxia-driven increase on the expression of SMO. These data suggest that GLI1 that directly mediated hypoxia-driven EMT process and invasion capacity of HCC cells.

It has been confirmed that hypoxia lead to an increased generation of ROS, which are important mediators of hypoxia-induced cellular response [9]. Increasing studies have confirmed a close relationship between ROS and EMT progress and cell invasion [41]. Our data demonstrated that hypoxia stimulated the accumulation of intracellular ROS. When the levels of ROS were inhibited by its inhibitor NAC, the GLI1-dependent EMT progress and cell invasion were also inhibited under hypoxia condition. These data showed that ROS was responsible for the hypoxia-driven GLI1-dependent EMT progress and aggressive capacity of HCC cells.

The NOX family members play a vital role in multiple cell biological progress, such as signal transduction, migration and proliferation. Previous study suggested that NOX members was an important initial factor of ROS affecting cell EMT phenotype and cell migration [14]. NOX4 is overexpression in multiple cancers, including HCC. NOX induced ROS production can activate STAT3 signaling pathway. In this research, we demonstrated that hypoxia promoted NOX4 expression. Moreover, NOX4 knockdown decreased the ROS generation in HCC cells exposed to hypoxia, which showed that NOX4 play a critical role in ROS accumulation in hypoxia. In addition, we also observed that the NOX4 knockdown significantly abolished hypoxia-induced GLI1-dependent EMT and invasion of HCC cells. Collectively, these data suggest that NOX4 plays a critical role in hypoxia-driven ROS-induced GLI1-dependent EMT progress and aggressive capacity of HCC cells.

Conclusion

Our data reveal that hypoxia triggers ROS-mediated GLI1-dependent EMT progress and aggressive capacity of HCC cells through induction of NOX4 expression. Thus, hypoxia-driven ROS mediated non-canonical Hh signaling may have a critical function in the initiation of EMT and provides a potential marker for HCC patients.

Acknowledgements

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ZKL, KST, YFW, BWY, MX, QL, LW, CWD, LLD, YLJ, and TS carried out the molecular experiment. XZ and QGL design the research. ZKL took part in its project, perform, data processing and paper writing.

Disclosure Statement

No conflicts of interest exist.

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