# Original Article ING5 inhibits hypoxia-induced epithelial-to-mesenchymal transition in human hepatocarcinoma cells via suppression of the HIF-1α/Notch signaling pathway

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**Abstract:** Hypoxia is one of the most common and critical factors identified in the regulation of epithelial-mesenchymal transition (EMT) in cancer cells. ING5 is a member of the inhibitor of growth (ING) candidate tumor suppressor family. However, the effect of ING5 in hypoxia-mediated EMT in hepatocellular carcinoma (HCC) cells is still unclear. Therefore, in this study, we sought to investigate the effect of ING5 on hypoxia-mediated EMT in HCC cells. We found that the expression of ING5 was lowly expressed in the HCC tissues and cell lines, and hypoxia significantly decreased the expression of ING5 in HepG2 cells. Moreover, forced expression of ING5 inhibited the expression of HIF-1 $\alpha$ , as well as the expression of Notch1 and Hes1 induced hypoxia in HepG2 cells. In conclusion, our results demonstrate that ING5 inhibits hypoxia-induced EMT in human HCC cells via suppression of the HIF-1 $\alpha$ /Notch signaling pathway. These results suggest a potential role for targeting ING5 in the prevention of hypoxia-induced HCC cancer progression and metastasis mediated by EMT.

**Keywords:** Inhibitor of growth (ING) 5, hepatocellular carcinoma (HCC), hypoxia, epithelial-mesenchymal transition (EMT)

#### Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and its incidence continues to rise [1]. Despite the advancements in therapeutic methods in recent years [2-4], the 5 year survival rate of patients with HCC has not improved because of early-stage metastasis, which involves the occurrence and the development of the epithelial-mesenchymal transition (EMT) [5].

During EMT progress, tumor cells lose epithelial E-cadherin expression and cellular adhesion, and acquire increased potential for local invasion and ability to evade to distant organs [6]. In solid tumors, hypoxic regions are commonly presented throughout the tumor tissue. Previous studies showed that hypoxia induces EMT in tumor cells. It could upregulate the expression of HIF-1, which binds to the promoter of TWIST1 and promote its transcript expression [7-9]. Therefore, preventing hypoxiainduced EMT is a promising approach for treatment of HCC.

The inhibitor of growth (ING) gene family includes ING1, ING2, ING3, ING4 and ING5. All ING proteins share a highly conserved carboxyterminal plant homeodomain (PHD) and control cell growth, senescence, apoptosis, DNA repair and chromatin remodeling in multiple systems of the body [10-13]. ING5 is a member of the ING family and has been induced the expression of p21/waf1 and interacts with Cyclin A1 inhibitor to suppress cell cycle progression [12]. Recently, down-regulation of ING5 was observed in a series of malignancies, including gastric cancer, bladder cancer, and lung cancer [14-16]. Upregulation of ING5 suppressed the proliferation, migration, invasion, and drug resistance in cancer cells. However, the effect of ING5 in hypoxia-mediated EMT in HCC cells is still unclear. Therefore, in this study, we sought to investigate the effect of ING5 on hypoxiamediated EMT in HCC cells.

## Materials and methods

## Tissue specimens

HCC tissue samples and their adjacent nontumorous liver tissues were obtained from 21 patients who underwent curative partial hepatectomy at the Department of Oncology, Huaihe Hospital of Henan University (China), during the period from 2013 to 2014. All HCC tissues were collected using protocols approved by the Ethics Committee of Huaihe Hospital of Henan University, and informed consent was obtained from all patients. All fresh samples were immediately frozen after resection and stored at -80°C until use.

# Cell culture and hypoxia treatment

Human HCC cell lines (HepG2, SMMC-7721 and BEL-7404) and a hepatocyte cell line (HL-7702) were purchased from the American Type Culture Collection (USA). These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA). Cells were exposed to hypoxia by placing them in a mixed-gas incubator that was infused with an atmosphere consisting of 94%  $N_2$ , 5% CO<sub>2</sub>, and 1% O<sub>2</sub> and then incubated for different hours as needed.

# Quantitative real-time reverse transcription PCR (qRT-PCR)

Total RNA of cells was isolated using the RNeasy Micro Kit (Qiagen, Valencia CA) and transcribed to complementary DNA (cDNA) with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, California). PCR was carried out with SYBR Green PCR Master Mix (Applied Biosystems) on StepOnePlus Real-Time PCR system (Applied Biosystems). The primers used for amplification were as follows: ING5, 5'-TCC AGA ACG CCT ACA GCA AG-3' (forward), and 5'-TGC CCT CCA TCT TGT CCT TC-3' (reverse); β-actin, 5'-GAT CAT TGC TCC TCC TGA GC-3' (forward), and 5'-ACT CCT GCT TGC TGA TCC AC-3' (reverse). For relative quantification, the levels of individual gene mRNA transcripts were firstly normalized to the control *β*-actin. Subsequently, the differential expression of these genes was analyzed by the DCt method and expressed as the fold changes.

# Western blotting

Total protein extracts were prepared using RIPA lysis buffer (Beyotime, Nantong, China) according to the operating instructions. The protein concentration in the lysates was evaluated using a BCA protein assay kit (Beyotime, Nantong, China). Total proteins were separated on a 10% polyacrylamide-SDS gel electrophoresis and transferred to nitrocellulose membranes (GE Healthcare, Milano, Italy) by electroblotting. Membranes were blocked with 5% non-fat dry milk in phosphate-buffered saline containing 0.1% Triton X-100 and incubated with primary antibodies (anti-ING5, anti-E-cadherin, anti-N-cadherin, anti-HIF-1α, anti-Notch1 and anti-Hes1) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. Then the blots were washed and incubated with horseradish peroxidase-conjugated secondary antibody. After washing, the sites of antibody binding were visualized by chemiluminescence (Boehringer Mannheim, Mannheim, Germany) and the relative levels of each protein to the GAPDH were analyzed.

# Transwell migration and invasion assay

For the migration assay, HepG2 cells at a density of 1×10<sup>5</sup> cells/well were suspended in serum-free medium and plated on chambers (Corning Costar, NY, USA) that were not coated with Matrigel. For the invasion assay, HepG2 cells at a density of 1×10<sup>5</sup> cells/well were seeded into the upper chamber that was precoated with Matrigel (BD Bioscience, CA, USA). For both assays, medium containing 10% FBS was added to the lower chamber as a chemoattractant. After incubating for 24 h at 37°C in the incubator supplemented with 5% CO<sub>2</sub>, the cells on the upper surface of the filters were removed with a cotton swab, and cells that had migrated to the lower surface were fixed with 100% methanol stained with 1% toluidine blue. After washing and desiccation, cells in each well were counted using a light microscope.

# Constructs and establishment of stable cell lines

To generate stable ING5 overexpression cells, breast cancer cells were infected with GV218-EGFP-ING5 lentivirus construct (Genechem, Shanghai, China). Single-cell clones were isolated by 5  $\mu$ g/ml puromycin for 48 h followed by 1  $\mu$ g/ml puromycin treatment. Empty vector-infected cells were used as control.

ING5 inhibits hypoxia-induced EMT in human HCC cells



**Figure 1.** ING5 was lowly expressed in HCC tissues and cell lines. A. The expression levels of ING5 mRNA were significantly decreased in HCC tissues compared with that in the corresponding normal liver tissues. B. ING5 protein expression was significantly decreased in HCC tissues compared with that in the corresponding normal liver tissues. C. Representative mRNA expression of ING5 in HCC cell lines. D. Representative Western blot image of ING5 protein in HCC cell lines. Data are expressed as mean ± SD. Experiments were performed in triplicate. \**P* < 0.05 compared with the control group.

#### Statistical analysis

All results are reported as means  $\pm$  SD. Statistical analysis involved using the Student's *t* test for comparison of 2 groups or 1-way ANOVA for multiple comparisons. *P* < 0.05 was considered to be significant.

#### Results

# ING5 was lowly expressed in HCC tissues and cell lines

First, we determined the expression of ING5 in HCC tissues and cell lines using RT-qPCR and Western blot. As indicated in **Figure 1A** and **1B**, as compared with the adjacent normal liver tis-

sues, the expression levels of ING5 mRNA and protein were significantly decreased in HCC tissues. Similarly, ING5 mRNA and protein expression were also decreased in HCC cell lines (**Figure 1C** and **1D**).

# Hypoxia decreases ING5 expression in HepG2 cells

To determine whether exposure to hypoxia affects ING5 expression, we exposed HepG2 cells to normoxia or hypoxia for 24 h and determined the mRNA levels of ING5 using RT-qPCR. We found that hypoxia inhibited mRNA level of ING5 in HepG2 cells (**Figure 2A**). Furthermore, we measured the protein levels of ING5 and



**Figure 2.** Hypoxia decreases ING5 expression in HepG2 cells. HepG2 cells were grown under hypoxia for 24 h. A. RT-qPCR analysis was performed for ING5.  $\beta$ -actin was used as an internal control. B. The expression of ING5 in HepG2 cells under hypoxia was examined using Western blotting. Data is expressed as mean ± SD. Experiments were performed in triplicate. \**P* < 0.05 compared with the normoxia group.

as indicated in **Figure 2B**, hypoxia greatly decreased the protein expression of ING5 in HepG2 cells.

#### ING5 overexpression inhibited hypoxia-induced EMT in HepG2 cells

To investigate the effect of ING5 on HepG2 cell migration and invasion, we forced expression of ING5 in HepG2 cells by stable transfection and selected with G418. The G418-resistant cells were pooled and examined the expression of exogenous ING5 (**Figure 3A**).

Previous studies demonstrated that hypoxia induces EMT in HepG2 cells and EMT has been involved in the promotion of cancer cell migration and invasion [17, 18]. So, we determined the effect of ING5 on hypoxia-mediated EMT in HepG2 cells. The expression levels of E-cadherin and N-cadherin in different groups were evaluated using Western blot. As shown in **Figure 3B**, hypoxia greatly downregulated the expression level of E-cadherin, and upregulated the expression level of N-cadherin in HepG2 cells. However, ING5 overexpression obvious prevented hypoxia-induced EMT process.

## ING5 overexpression suppresses hypoxiainduced HepG2 cell migration and invasion

Since hypoxia is known to stimulate cell migration and invasion, thus, we investigated the effect of ING5 on cancer cell migration and invasion under hypoxia condition. As shown in **Figure 4A**, hypoxia greatly increased the number of migrated cells, whereas, ING5 overexpression prevented hypoxia-induced migration in HepG2 cells. We determined cell invasion using invasion chambers coated with matrigel. As shown in **Figure 4B**, ING5 overexpression prevented hypoxia-mediated cell invasion.

# ING5 overexpression restrains the activation of HIF-1 $\alpha$ /Notch signaling pathway induced by hypoxia in HepG2 cells

HIF-1 plays an important role in HCC progression by increasing the expression of several target genes involved in invasion [19]. So, we determined the effect of ING5 on HIF-1 $\alpha$  in HepG2 cells under hypoxia condition. As shown in **Figure 5A**, HepG2 cells responded to hypoxia with a clear accumulation of HIF-1 $\alpha$ , and ING5 overexpression was also able to restrain HIF-1 $\alpha$  expression under hypoxia exposure.

Several researches have shown that Notch signaling is necessary for coupling hypoxia to the EMT [20-22]. Therefore, we investigated the effect of ING5 on Notch pathway components expression under hypoxia condition. As shown in **Figure 5B**, hypoxia significantly increased the expression of Notch1 and Hes1 in HepG2 cells. In addition, ING5 overexpression was also able to prevent Notch1 and Hes1 upregulation under hypoxia exposure.

## Discussion

In this study, the expression of ING5 was found to be down-regulated in the HCC tissues and



**Figure 4.** ING5 overexpression suppresses the migration and invasion of HepG2 cells. HepG2 cells were transfected with Ad-ING5 or Ad-GFP, and cultured under normoxia or hypoxia for 24 h. A. Cell migration was determined by the Transwell assay. B. Cell invasion was determined using Matrigel-coated transwell invasion chamber. Data represents means  $\pm$  SD of three independent experiments; \*P < 0.05 vs. normoxia group; &P < 0.05 vs. hypoxia group.

cell lines, and hypoxia significantly decreased the expression of ING5 in HepG2 cells.

Moreover, forced expression of ING5 inhibited hypoxia-induced the migration/invasion and



**Figure 5.** ING5 overexpression restrains the activation of HIF-1 $\alpha$ /Notch signaling pathway induced by hypoxia in HepG2 cells. HepG2 cells were transfected with Ad-ING5 or Ad-GFP, and cultured under normoxia or hypoxia for 24 h. A. Cell lysates were subjected to Western blot analysis to determine the expression of HIF-1 $\alpha$ . B. Cell lysates were subjected to Western blot analysis to determine the expression of HIF-1 $\alpha$ . B. Cell lysates were subjected to Western blot analysis to determine the expression of HIF-1 $\alpha$ . B. Cell lysates were subjected to Western blot analysis to determine the expression of Notch1 and Hes1. Data represent means ± SD of three independent experiments; \*P < 0.05 vs. normoxia group; &P < 0.05 vs. hypoxia group.

EMT process in HepG2 cells. In addition, forced expression of ING5 inhibited the expression of HIF-1 $\alpha$ , as well as the expression of Notch1 and Hes1 induced hypoxia in HepG2 cells.

Several studies suggest a role for ING5 in cancer development. Deregulation of ING5 is observed in different tumors, potentially functioning as a tumor suppressor [14-16]. In this study, we demonstrated for the first time that ING5 is lowly expressed in human HCC tissues and cell lines. Hypoxia is an important microenvironmental pressure present in the majority of solid tumors [23]. We found that hypoxia decreased the expression of ING5 protein and mRNA, suggesting that ING5 may be a tumor suppressor in HCC.

EMT is characterized by loss of cell adhesion, repression of E-cadherin expression and increased cell mobility [24]. Solid tumors often contain regions with insufficient oxygen delivery, a condition known as hypoxia, and emerging studies have indicated that hypoxia induces EMT in various cancers, including HCC [18, 25-27]. In line with these results, we found that hypoxia induced the migration/invasion and EMT process in HepG2 cells, whereas, forced expression of ING5 inhibited hypoxia-induced these effects. These results suggest that ING5 inhibits hypoxia-induced EMT, consequently affects cell migration and invasion *in vitro*.

HIF is composed of  $\alpha$  subunit and  $\beta$  subunit. Under normoxic conditions, HIF-1 $\alpha$  is targeted for proteasomal degradation by prolyl hydroxylase domain (PHD) proteins, whereas under hypoxia, HIF-1 $\alpha$  is able to enter the nucleus and bind to HIF-1 $\alpha$  to coordinate gene expression of target genes [28]. It was reported that pharmacologic blockade of HIF-1 $\alpha$  protein expression using digoxin significantly suppressed cellular invasion in hypoxia [29]. In this study, we found that hypoxia greatly increased the expression of HIF-1 $\alpha$ , and ING5 overexpression was also able to restrain HIF-1 $\alpha$  expression under hypoxia exposure.

Notch signaling plays important roles in regulating cancer cell proliferation, differentiation, invasion and apoptosis [30-33]. Notch signaling is activated by ligand-receptor binding between the neighboring cells. Once activated, Notch-1 is cleaved by c-secretase and releases the Notch-1 intracellular domain (NICD) from the plasma membrane, which then translocates into the nucleus to engage other transcription factors and regulate the expression of target genes including some members of Hes and Hey gene families. A growing body of evidence indicates that Notch signaling was closely related with hypoxia [34-36]. For example, one study showed that inhibition of Notch signaling abrogated hypoxia-induced EMT and invasion, and, conversely, an activated form of Notch could substitute for hypoxia to induce these processes in ovarian carcinoma cell line SKOV-3 [37]. Furthermore, previous studies have shown that HIF-1 $\alpha$  can directly stabilize the activated Notch1 intracellular domain (NICD1), thereby increasing signaling in hypoxia [37]. Chen et al. reported that Notch pathway components, including Jag1-2 ligands, Hes1-Hey1 targets and the intracellular domain of Notch1 were increased in hypoxia [38], and pharmacologic and genetic inhibition of Notch largely blocked the hypoxic induction of invasion in uveal melanoma cells [29]. These data suggest that pharmacologically targeting HIF-1α pathway may through blockade of Notch signaling pathway can suppress tumor spread. In line with these results, in this study, we observed that hypoxia significantly increased the expression of Notch1 and Hes1 in HepG2 cells. Our findings are consistent with the observation of elevated Notch signaling during hypoxia in many different tumors [37, 39]. Moreover, we found that ING5 overexpression suppressed Notch1 and Hes1 upregulation under hypoxia exposure. These results suggest that ING5 overexpression inhibits HCC cell migration and invasion through the regulation of the EMT dependent on Notch signaling pathway.

In conclusion, our results demonstrate that ING5 inhibits hypoxia-induced EMT in human HCC cells via suppression of the HIF-1 $\alpha$ /Notch

signaling pathway. These results suggest a potential role for targeting ING5 in the prevention of hypoxia-induced HCC cancer progression and metastasis mediated by EMT.

# Disclosure of conflict of interest

None.

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