Overexpression of interleukin-32α promotes invasion by modulating VEGF in hepatocellular carcinoma

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Abstract. Interleukin-32α (IL-32α) was reported to exhibit pluripotent pro-inflammatory properties. Recent studies indicate that it promotes the migration and invasion of cancers. We detected the expression of IL-32 in hepatocellular carcinoma (HCC) tissues and investigated its role in tumor angiogenesis and invasion. IL-32α expression in HCC was evaluated by real-time PCR, western blot analysis and immunohistochemical (IHC) staining. Secreted serum IL-32α and VEGF concentrations were detected using a custom-made sandwich ELISA. Furthermore, IL-32α was knocked down in HCC cell lines using siRNA and the cell migration and invasion abilities were assessed. IHC staining showed that IL32α-positive particles were mainly located in the cytoplasm of cancer cells, and it was significantly upregulated in the tumor tissues compared with that in peritumoral tissues. Notably, IL-32α was strongly expressed in perivascular areas. The mean serum concentration of IL-32α in HCC patients was significantly higher than that in the control group (571.45±102.28 vs. 144.60±51.172 pg/ml; P<0.01). Real-time RT-PCR showed that IL-32α mRNA was significantly overexpressed in HCC tumor tissues (IL-32/β-actin, 15.59±7.8 vs. 3.37±0.47; P<0.01). The *in vitro* results indicated that IL-32α knockdown inhibited the activation of VEGF-STAT3 signaling in HCC tumor cell lines. IL-32α expression was correlated with clinical relevance in HCC tumor tissues. It is strongly suggested that IL-32α may be a potential predictor of anti-angiogenesis therapy and prognosis of HCC.

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Abbreviations: HCC, hepatocellular carcinoma; IHC, immunohistochemistry; IL-32, interleukin-32; NK, natural killer; VEGF vascular endothelial growth factor; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; ELISA, enzyme-linked immununosorbent assay

Key words: hepatocellular carcinoma, IL-32α, VEGF, angiogenesis

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide and the third most common cause of cancer-related death (1,2). It is particularly prevalent in Asia and sub-Saharan Africa countries (3,4). A progressive increase in HCC-related mortality has been observed in the US and Western Europe (5-7). Accumulating evidence has revealed that inflammatory-related cytokines participate in the carcinogenesis and progression of HCC (8). Research has revealed that higher expression of interleukin-32 (IL-32), a novel pro-inflammatory cytokine, is detected in HCC (9). However, the potential roles of IL-32 in the carcinogenesis and progression of HCC remain unclear.

IL-32, originally called natural killer (NK) cell transcript 4, is a recently described cytokine that is mainly produced by T, NK and epithelial cells after stimulation (10,11). Six splice variants have been reported in the IL-32 family, including IL-32α, IL-32β, IL-32δ, IL-32γ, IL- 32ε and IL-32ζ (12). Besides its pluripotent pro-inflammatory properties, it has been unambiguously shown that IL-32α enhances the migration and invasion of cancers, such as breast cancer, gastric cancer and lung cancer (13-15). However, the function and role of IL-32α in HCC progression remain unknown.

The present study explored the expression of IL-32α in HCC and its role in vascular invasion and tumor progression. Mechanistic investigation was conducted to show the potential downstream factor in the IL-32 signaling pathway. The results suggested a specific mechanism of IL-32 in HCC and present a potential therapeutic target for HCC treatment and drug development.

Materials and methods

Patients and tissue specimens. Tumor tissues and paired non-cancerous hepatic parenchyma were collected from 100 patients with primary HCC who received surgical resection from May 2010 to June 2011 at the Department of Hepatobiliary Surgery, Shandong Provincial Hospital Affiliated to Shandong University. Serum specimens were collected from the patients and 30 control patients without HCC. None of the patients had

received preoperative chemotherapy or other treatment before surgery. Patient written informed consent was obtained, and the study protocol was approved by the Health Service Ethics Committee of Shandong Provincial Hospital Affiliated to Shandong University. HCC was histologically diagnosed by two pathologists independently and the clinical characteristics of each patient were recorded as shown in Table I.

Ethical approval. The present study was performed in accordance with the Declaration of Helsinki and approved by the local Ethics Committee. All patients provided their informed consent.

Quantitative real-time RT-PCR. Fresh HCC tissues were treated with TRIzol reagent for total RNA extraction (Invitrogen Carlsbad, CA, USA) and purified by phenol/$CHCl_3$ according to the manufacturer's instructions. Total RNA (5 μg) was reversely transcribed to cDNA using the MBI Fermantas reverse transcription kit (MBI Fermentas, Vilnius, Lithuania). The Quantitative SYBR-Green PCR kit and ABI Prism 7000 Sequence Detection System (both from ABI, USA) were applied to test the expression level of IL-32α under the following conditions: 30 cycles: 1 cycle at 95˚C for 5 min, then 30 cycles at 94˚C for 30 sec and 60˚C for 45 sec; quantitative RT-PCR was repeated at least 3 times. β-actin expression was used for normalization. Primer sequences are listed as follows: IL-32α F, 5'-ACAGTGGCGGCTTATTATGAGGA-3' and R, 5'-GTTGCCTCGGCACCGTAATC-3'; β-actin F, 5'-AATGC TTCTAGGCGGACTATGA-3' and R, 5'-CAAGAAAGGGT GTAACGCAACT-3'.

Western blotting. Five fresh HCC and paired non-cancerous tissues were lysed by cold RIPA buffer containing protease inhibitor on ice for 30 min, and centrifuged at 12,000 x g at 4˚C for 20 min. The protein concentration was determined using a BCA protein assay kit (Biocolor Biotech, Shanghai, China). Proteins suspended in loading buffer were denatured and separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA) in transfer buffer at 40 V for 105 min. The membrane was blocked using 5% skimmed milk in Tris-buffered saline with Tween-20 (TBST) for 2 h, washed with TBST and incubated with mouse anti-IL-32 antibody (R&D Systems, Minneapolis, MN, USA) overnight at 4˚C. The membrane was incubated with an anti-mouse horseradish peroxidase-conjugated secondary antibody (Dako, Glostrup, Denmark) at a dilution of 1:200 at room temperature for 1 h. Protein bands were visualized by SuperSignal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, IL, USA) and exposed using Kodak X-ray film (Kodak, Rochester, NY, USA). Proteins were re-blotted with anti-GAPDH (Zymed, South San Francisco, CA, USA) as an internal control.

Immunohistochemical staining. For immunohistochemical analysis, 4-μm tissue sections were cut from paraffin blocks and baked at 60˚C for 2 h before staining with mouse anti-IL-32α antibody (dilution 1:100; R&D Systems). Endogenous peroxidase activity was blocked with 3% H_2O_2 for 30 min. Then tissue sections were pre-treated in citrate buffer using a water bath for 15 min for antigen retrieval. Goat serum (1%) was applied to prevent a non-specific reaction. The primary antibody was incubated overnight at 4˚C. An anti-mouse antibody kit (Jing Mei Biotech, Shanghai, China) was applied and DAB reaction was performed following the protocol. Control IgG antibody was used as a negative control. Histomorphometric analysis was performed by Image-Pro Plus image analysis system (Media Cybernetics, Inc., Rockville, MD, USA).

Table I. Correlations between serum IL-32 expression and clinicopathological parameters in 100 patients with HCC.

Clinicopathological parameters	Cases	T/N[a] (mean ± SE)	P-value
Age (years)			0.312[b]
<60	72	16.49±9.10	
≥60	28	13.50±3.03	
Sex			0.184[b]
Male	80	15.78±4.88	
Female	20	13.56±3.20	
Virus			
HBV	54	14.48±3.56	0.271[c]
HCV	6	14.31±4.96	
None	40	16.64±5.72	
AFP (ng/ml)			0.157[b]
<20	30	13.89±3.33	
≥20	70	15.96±5.02	
Tumor multiplicity			0.249[b]
Single	90	15.07±4.70	
Multiple	10	17.69±3.72	
Tumor size (cm)			0.460[b]
<3.5	46	14.80±4.03	
≥3.5	54	15.79±5.15	
Differentiation			0.798[c]
Well	16	14.42±3.45	
Moderate	60	15.35±5.10	
Poor	24	15.91±4.35	
Liver cirrhosis			0.811[b]
Yes	40	15.14±3.95	
No	60	15.47±5.12	
Vascular invasion			0.007
Yes	18	19.05±5.99[b]	
No	82	14.52±3.94	
Metastasis			0.011[b]
Yes	24	18.26±5.85	
No	76	14.41±3.84	

T/N[a], T/N ratio, IL32α/β-actin in tumor (T) divided by IL32α/β-actin in non-cancerous tissue (N). [b]P-value determined by the Mann-Whitney U test. [c]P-value determined by the Kruskal-Wallis test. HBV, hepatitis B virus; HCV, hepatitis C virus; AFP, α-fetoprotein; SE, standard error.

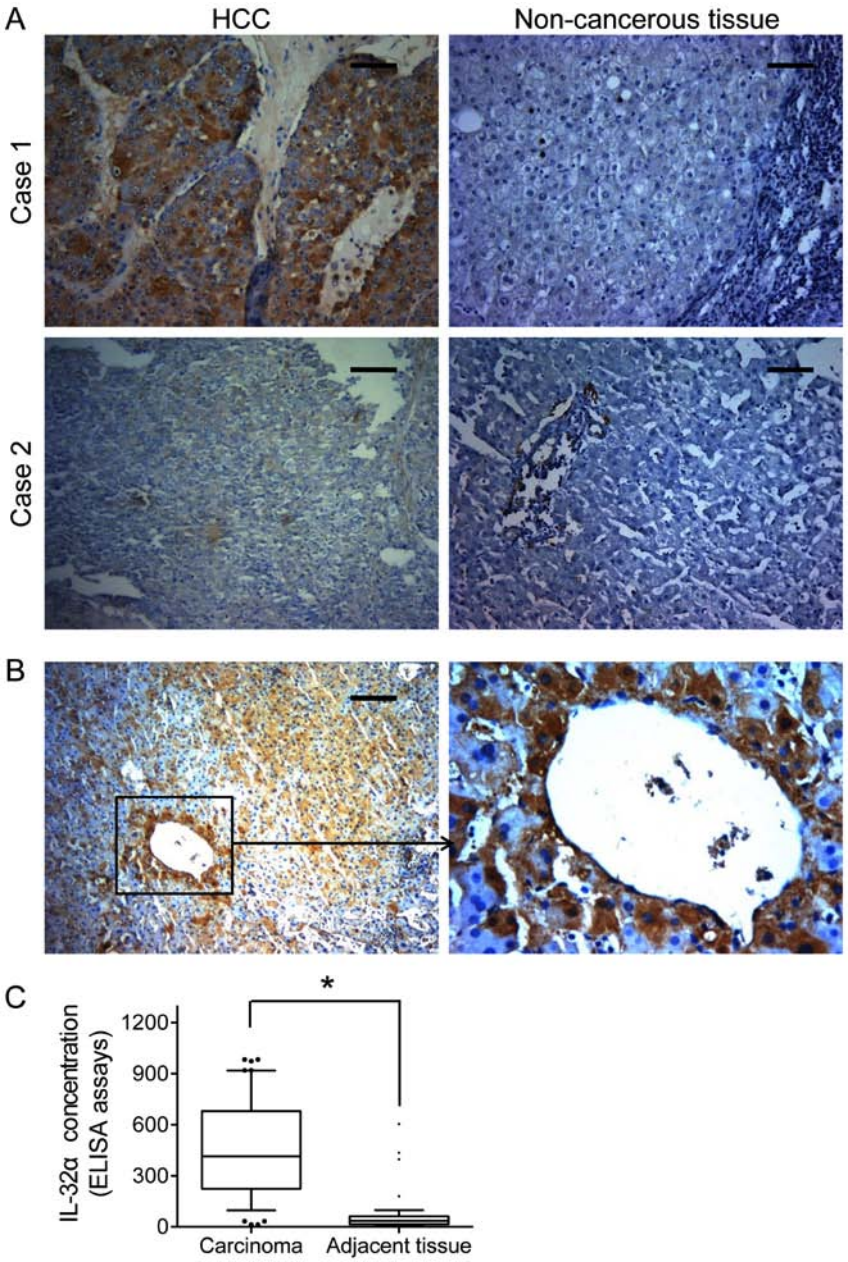

Figure 1. IL-32α overexpression in HCC correlates with vascular invasion. (A) Representative IHC images of IL-32α expression in HCC and paired non-cancerous tissues. Higher IL-32α was detected in cancerous tissues. Scale bar, 50 μm. (B) IHC staining images showed that IL-32α-positive cells were distributed in vessels of tumor tissues. (C) The mean serum concentration of IL-32α in HCC patients was significantly higher than that in the healthy control group, which was detected by ELISA assays; *P<0.05.

Enzyme-linked immununosorbent assay (ELISA). A sandwich ELISA was designed for the quantification of IL-32α in human serum. A 96-well microtiter plate was coated overnight at 4˚C with goat antibody (PAb; R&D Systems) to IL-32α (1 μg/ml in PBS, 100 μl/well) and rinsed with PBST. The wells were then coated with 1% BSA solution in PBS. IL-32α standard samples were prepared using a serial dilution of a recombinant human IL-32α solution. Samples were grouped into control and HCC. IL-32α ELISA was carried out according to the manufacturer's instructions as follows: assay diluent (80 μl) was added in duplicate to all wells. Each prepared standard dilution (20 μl) was added to samples and incubated at room temperature. Biotin-conjugate (100 μl) was added to all wells and incubated at room temperature. Diluted streptavidin-HRP (100 μl) was added to all wells and incubated at room temperature. The enzyme reaction was stopped by quickly pipetting 100 μl of stop solution into each well. Absorbance of the reaction product was measured at 490 nm on an ELISA reader (Molecular Devices, Sunnyvale, CA, USA).

Cell culture and siRNA transfection. HCC cell lines Hu7 and HepG2 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 25 ng/ml amphotericin B, and 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) at 37˚C in a humidified incubator with 5% CO_2. For the RNA interference assay, an siRNA for IL-32α was designed to silence IL-32α expression in HCC cell lines, Hu7 and HepG2 (Santa Cruz Biotechnology,

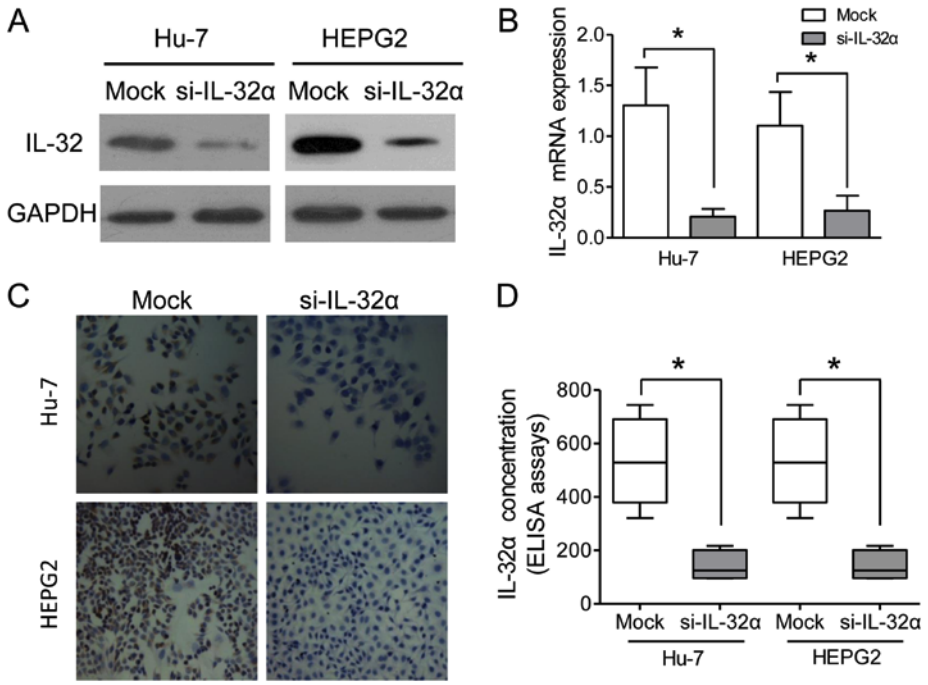

Figure 2. IL-32α silencing experiment. (A) IL-32α expression in Hu-7 cells (left) and HEPG2 cells (right) was knocked down with specific shRNA infection, which was detected by immunobloting assays. GAPDH was used as a loading control. (B) IL-32α mRNA levels in Hu7-si-IL-32α and mock cells and HEPG2-si-IL-32α and mock cells were examined by RT-PCR assays. β-actin was set as a control; *P<0.05. (C) Significantly decreased expression of IL-32α was observed in Hu7-si-IL-32α cells compared with mock cells (above) and HEPG2-si-IL-32α cells compared with mock cells (below), which was detected by cell IHC staining. (D) Similar concentrations of cells (si-IL-32α and mock cells of Hu7 or HEPG2) were cultured for one day. ELISA assays were also conducted to test IL-32 secretion in the culture medium. Significantly decreased IL-32α level was observed in Hu7-si-IL-32α cells; *P<0.05.

Santa Cruz, CA, USA). The cells were transfected with 40 nM of siRNA using Lipofectamine™ LTX (Invitrogen). Silencing efficiency was verified by western blot analysis. Exogenous IL-32α at a similar concentration (500 pg/ml) was added to the culture medium for the rescue assay.

Detection of invasion and migration by scratch and Transwell assays. Transfected and control cells were subjected to cell scratch and Transwell invasion assays. For the scratch assay cells were seeded into 6-well plates and cultured until reaching confluence. A wound was created with a sterile pipette tip. The distance was measured by a Nikon DS-5M Camera System mounted on a phase-contrast Leitz microscope. Images of the wound were captured under a phase-contrast microscope at 0, 24 and 48 h. For each experiment, 5 visual fields and 2 repeated wells were measured with 3 replications.

For the Transwell assay a 24-well Transwell chamber (8-mm; Millipore) coated with 30 μl Matrigel was used for the invasion assay. A 100-μl cell suspension was loaded into the upper Matrigel-coated chamber. DMEM (600 ml) with 10% FBS was added to the bottom chamber. Cells were then allowed to migrate or invade for 48 h at 37˚C. The cells in the bottom chamber were fixed in paraformaldehyde and permeabilized in methanol, and then stained with crystal violet dye. Cell images were obtained under a light microscope (Leica DM4000 B; Leica Microsystems, Wetzlar, Germany.

Statistical analysis. The Mann-Whitney U test or Kruskal-Wallis was used for between-group comparisons, where appropriate, and the correlation between the results obtained with the two different analyses was analyzed with the Spearman's test. A paired Student's t-test was used to compare the differences of IL-32-α mRNA and protein expression in tumor tissues and non-cancerous tissues. The correlations of mRNA expression levels were analyzed with Pearson test. P<0.05 was considered statistically significant. All data were analyzed with SPSS 16.0 (SPSS, Inc., Chicago, IL, USA).

Results

IL-32α overexpression in HCC correlates with vascular invasion. In order to investigate the expression pattern of IL-32α in HCC tissue and its prognostic role in HCC patients, we examined the IL-32α expression levels in 100 HCC samples. IL-32α expression was found widely elevated in the HCC tissues, as compared with that in the paired non-cancerous tissues (Fig. 1A). Moreover, we analyzed the correlation between IL-32α serum levels of HCC patients and clinicopathological parameters, including tumor size, virus infection, liver cirrhosis, vascular invasion and metastasis. Statistical results revealed that IL-32α was much higher in the serum samples of patients with distant metastasis than those without distant metastasis and in patients with vascular invasion (Table I; P=0.01). Similarly, IHC staining revealed that high IL-32α expression was often observed in vessel invasion foci (Fig. 1B). Importantly, HCC patients showed a higher IL-32α serum concentration than the controls (571.45±102.28 vs. 144.60±51.172 pg/ml, P=0.007, Fig. 1C). Taken together, these findings suggest that IL-32α overexpression can serve as a predictive indicator for distant metastasis and vascular invasion of HCC patients.

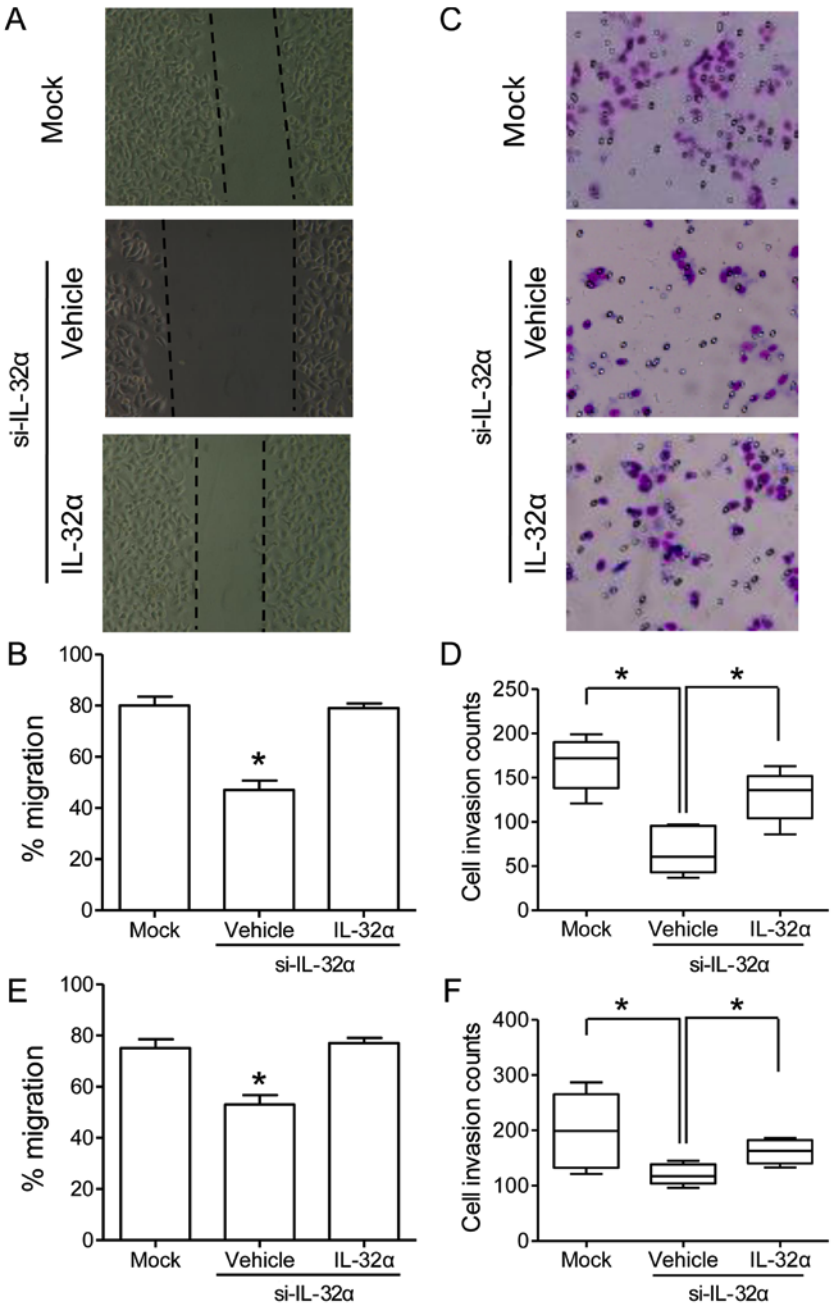

Figure 3. IL-32α promotes the migration and invasion of HCC cells. (A) Cell scratching assays showed impaired migration ability in the Hu7-si-IL-32α cells when compared with the mock cells. The top image is performed with mock cells. The bottom 2 images were IL-32α-knockdown cells, and vehicle and exogenous IL-32α was added for rescue. (B) Transwell invasion assays revealed impaired invasive potential of Hu7-si-IL-32α cells as compared with that of the Hu7-mock cells; *P<0.05. (C) Transwell invasion assays showed that IL-32α treatment restored the migration ability of Hu7-si-IL-32α cells. Exogenous IL-32α (500 pg/ml) was added to the culture medium. (D) Exogenous IL-32α (500 pg/ml) treatment restored the invasion ability of Hu7-si-IL-32α cells, which was revealed by Transwell invasion assays; *P<0.05. (E) Cell scratching assays showed sharply reduced migration ability in HepG2-si-IL-32α cells than mock cells. IL-32α treatment restored migration ability of HepG2-si-IL-32α cells. Exogenous IL-32α (500 pg/ml) was added in the culture medium; *P<0.05. (F) Transwell invasion assays revealed the decreased invasive potential of HepG2-si-IL-32α cells than that of HepG2-mock cells. Exogenous IL-32α (500 pg/ml) treatment restored invasion ability of HepG2-si-IL-32α cells; *P<0.05.

IL-32α promotes migration and invasion of HCC cells. In order to verify the functions of IL-32α in HCC *in vitro*, an siRNA of IL-32α was designed to silence the IL-32α expression in HCC cell lines, Hu7 and HepG2. The results indicated that IL-32α was significantly downregulated in the si-IL-32α-treated Hu7 and HepG2 cells, as compared with the mock group (Fig. 2A and B). IHC staining assays also confirmed the knockdown of IL-32α in the HepG2 Hu7 cells (Fig. 2C). As a secreted factor, IL-32α was also detected in the culture medium, and its concentration in the supernatant was decreased after siRNA knockdown (Fig. 2D). Cell scratch and Transwell invasion assays were carried out in order to examine the cell migration and invasion abilities, respectively. Cell scratch assays revealed that Hu7-si-IL-32α cells showed sharply reduced migration ability as compared with that of mock cells (Fig. 3A and B; P<0.05). For Transwell invasion assays, Hu7-si-IL-32α cells showed decreased invasive potential than that of Hu7-mock cells (Fig. 3C and D; P<0.05). Similar results

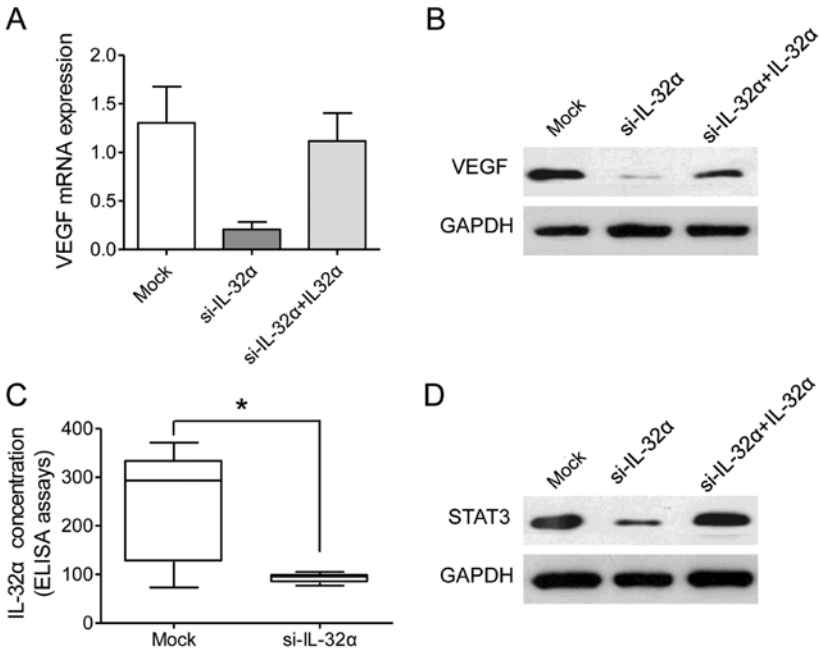

Figure 4. IL-32α regulates VEGF in HCC cells. (A and B) Significantly reduced VEGF was observed in Hu7-si-IL-32α cells at the (A) mRNA and (B) protein level. GAPDH was set as a control. (C) ELISA assays showed that IL-32α silencing in Hu7 cells reduced secretion of VEGF in culture medium; *P<0.05. (D) Western blotting showed that STAT3 was significantly reduced in Hu7-si-IL-32α cells when compared with the mock cells. In addition, exogenous IL-32α (500 pg/ml) treatment increased the level of STAT3 in the Hu7-si-IL-32α cells. GAPDH was used as control.

were also observed in HepG2 cells both in cell scratching and Transwell invasion assays (Fig. 3E and F). To further confirm the role of IL-32α in regulating cell migration and invasion, exogenous IL-32α at a similar concentration (500 pg/ml) was added to the culture medium. Cell scratch assays showed that Hu7-si-IL-32α cells restored the migration ability with IL-32α treatment (Fig. 3A and B; P<0.05). For Transwell invasion assays, Hu7-si-IL-32α cells also displayed elevated invasive potential after IL-32α treatment (Fig. 3C and D; P<0.05). To sum up, these results indicated that IL-32α could positively regulate the migration and invasion ability of HCC cells.

IL-32α regulates VEGF in HCC cells. It has been reported that IL-32α regulates VEGF levels in breast cancer, which is linked to angiogenesis and tumor invasion (14,16). In order to test whether IL-32α also modulates VEGF in HCC cells, we examined the level of VEGF after IL-32α was transiently silenced in the Hu7 and HepG2 cell lines. The present study found that VEGF was significantly reduced after IL-32α knockdown at both the mRNA level and protein level (Fig. 4A and B). Furthermore, a decreased VEGF level in the culture medium was observed after IL-32α knockdown (Fig. 4C; P<0.05). Collectively, these data showed that VEGF was a downstream response factor of IL-32α in HCC cells.

Previous reports have revealed that VEGF-STAT3 signaling is important for vascular invasion in a series of tumors (17,18). In the present study, we provided further proofs for the correlation between IL-32α and VEGF-STAT3 signaling. STAT3 was significantly reduced in the Hu7-si-IL-32α cells than that noted in the mock cells (Fig. 4D). However, exogenous IL-32α treatment increased the level of STAT3 in the Hu7-si-IL-32α cells (Fig. 4D). Taken together, our data revealed that the IL-32α/VEGF/STAT3 signaling pathway plays an essential role in the vascular invasion in HCC.

IL-32α is positively correlated with VEGF in both HCC tissues and serum. To further confirm the correlation between IL-32α and VEGF in HCC, VEGF staining was performed on the HCC tissues and corresponding non-cancerous liver tissues. Our result verified that VEGF expression levels were in accordance with IL-32α in the HCC tissues, whereas their expression was low in paired non-cancerous tissues (Fig. 5A). Moreover, western blotting of IL-32α and VEGF were also conducted in 6 cases of HCC and corresponding non-cancerous liver tissues. A significant correlation was also detected between VEGF and IL-32α at the relative protein level (VEGF greyscale/GAPDH greyscale and IL-32α greyscale/GAPDH greyscale) (Fig. 5B). Protein level analysis of the western blotting revealed that IL-32α and VEGF levels were positively correlated (P<0.05; Fig. 5C). Furthermore, we investigated IL-32α and VEGF levels in the HCC patient serum samples. IL-32α and VEGF protein were positively correlated (Fig. 5D; P<0.05). Taken together, these data provide further proof that VEGF may serve as a downstream factor regulated by IL-32α in HCC.

Discussion

In the present study, we investigated the expression pattern and functions of IL-32α in HCC tissues. We demonstrated that elevated IL-32α in HCC tissues was correlated with the patient tumor stage as well as vascular invasion. We also revealed that silencing of IL-32α in HCC cells impaired the tumor migration and invasion properties. Importantly, we found that VEGF, an essential factor for cancer growth, invasion and metastasis, served as a downstream response of the IL-32α signaling pathway in HCC cells.

Increasing evidence has confirmed that inflammation plays a crucial role in liver carcinogenesis. Elevated inflammatory-related cytokines are commonly observed in

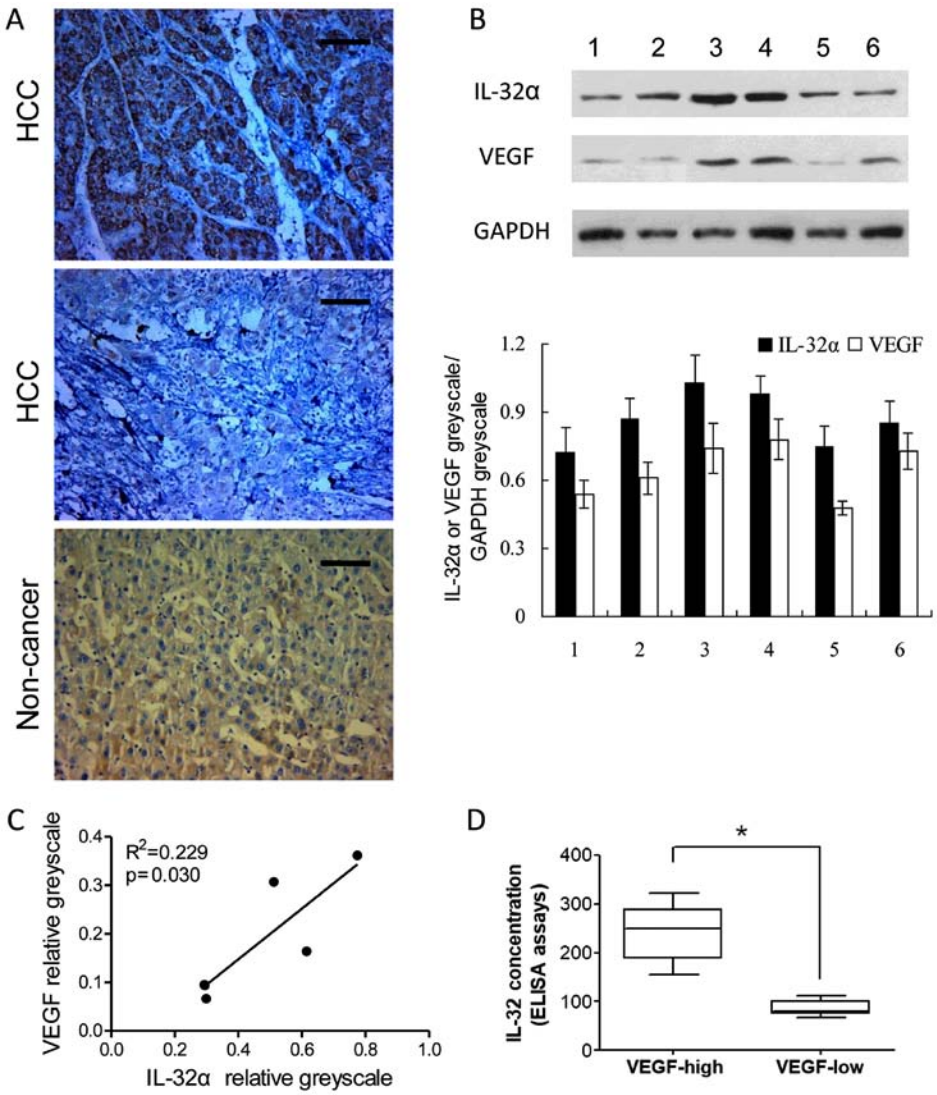

Figure 5. IL-32α is positively correlated with VEGF in HCC tissue and serum. (A) IHC staining of VEGF expression in HCC and paired non-cancerous tissues, which was consistent with IL-32α. Scale bar, 50 μm. (B) Western blotting revealed a significant correlation between VEGF and IL-32α relative protein level in tumor tissues (VEGF/GAPDH and IL-32α/GAPDH grayscale). GAPDH was used as the control. (C) Grey level analysis revealed that IL-32α and VEGF levels were positively correlated. (D) ELISA assays showed positive correlation between IL-32α and VEGF in serum; *P<0.05.

the carcinogenesis and progression of HCC (8,19,20). IL-32 is known as a pro-inflammatory cytokine since it enhances the production of IL-1β and TNFα (11,21). Higher expression of IL-32 in tumor tissues was observed compared with normal tissue or serum (22). However, different roles are observed with respect to the tumor types among the 6 members of the IL-32 family (23,24). IL-32α exhibits significant effects in human inflammatory disorders and cancers, and may be involved in the pathogenesis and progression from inflammation to cancer (25).

IL-32α expression has been observed in a series of tumor tissues, including gastric (26), breast (16) and esophageal cancer (27). Accumulated evidence indicates that IL-32α participates in cell proliferation and predicts patient overall outcome. IL-32α knockdown was found to inhibit cell growth and induce intrinsic apoptosis by decreasing phospho-p38, MAPK, NF-κB and Bcl-2, but increasing pro-apoptotic proteins, p53 and PUMA (19,28). Quite consistent with these studies, we found that IL-32α was elevated in HCC tissues and associated with patient metastasis as well as vascular invasion, which was reported for the first time. *In vitro* experiments provided convincing evidence that silencing of IL-32α in HCC cells sharply reduced the migration and invasion properties of HCC cell lines, which was correlated with VEGF-STAT3 signaling. Further studies will be conducted to investigate the functional role of IL-32α in HCC progression.

For HCC patients, tumor angiogenesis contributes to a poor therapy response and progression of residual disease (29). Among the tumor angiogenesis regulators, VEGF, an essential growth factor for cancer progression, invasion and metastasis, plays vital roles (30). Previous studies suggest IL-32 as a critical regulator of endothelial cell functions, which possesses angiogenic properties (31,32). Secreted VEGF was also found to be altered along with a change in IL-32α in breast cancer cells (14,17). Moreover, IL-32α induced VEGF increased migration and invasion through STAT3 activation, which is a potential target for HCC therapy (33,34). We demonstrated that VEGF is a downstream factor for IL-32α signaling in

HCC cells. The detailed mechanism by which IL-32α regulates VEGF expression requires further investigation.

In conclusion, our findings provide evidence for the clinical relevance and function of IL-32α in HCC. Elevated IL-32α in clinical specimens is predictive of tumor metastasis and vascular invasion in HCC patients, which was correlated with VEGF/STAT3 signaling, IL-32α is a promising therapeutic target for HCC treatment or drug development.

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