Research Article

JOURNAL OF HEPATOLOGY

EDIL3 is a novel regulator of epithelial-mesenchymal transition controlling early recurrence of hepatocellular carcinoma

Hongping Xia[1], Jianxiang Chen[1], Ming Shi[2], Hengjun Gao[2], Karthik Sekar[1], Veerabrahma Pratap Seshachalam[1], London Lucien P.J. Ooi[3,4], Kam M. Hui[1,5,6,7,*]

[1]*Laboratory of Cancer Genomics, Division of Cellular and Molecular Research, Humphrey Oei Institute of Cancer Research, National Cancer Centre, Singapore;* [2]*Department of Hepatobiliary Oncology, Cancer Center, Sun Yat-sen University, Guangzhou 510060, PR China;* [3]*Department of Surgical Oncology, National Cancer Centre, Singapore;* [4]*Department of General Surgery, Singapore General Hospital, Singapore;* [5]*Cancer and Stem Cell Biology Program, Duke-NUS Graduate Medical School, Singapore;* [6]*Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, Singapore;* [7]*Institute of Molecular and Cell Biology, A*STAR, Biopolis Drive Proteos, Singapore*

Background & Aims: Patients with advanced hepatocellular carcinoma (HCC) continue to have a dismal prognosis. Early recurrence, metastases and angiogenesis are the major obstacles to improve the outcome of HCC. Epithelial-mesenchymal transition (EMT) is a key contributor to cancer metastasis and recurrence, which are the major obstacles to improve prognosis of HCC.

Methods: Combining gene expression profiles of HCC samples with or without early recurrence and established cell lines with epithelial or mesenchymal phenotype, EDIL3 was identified as a novel regulator of EMT. The expression of EDIL3 was evaluated by quantitative PCR, Western blotting or immunohistochemistry. The effects of EDIL3 on the angiogenesis and metastasis of HCC cells were examined by wound healing, Matrigel invasion and tube formation assay *in vitro* and orthotopic xenograft mouse model of HCC *in vivo*. The signaling pathways of EDIL3 mediated were investigated through microarray and Western blotting analysis.

Results: EDIL3 was identified as a novel regulator of EMT, which contributes to angiogenesis, metastasis and recurrence of HCC. EDIL3 induces EMT and promotes HCC migration, invasion and angiogenesis *in vitro*. Mechanistically, overexpression of EDIL3, which was regulated by the downregulation of miR-137 in HCC, triggered the activation of ERK and TGF-β signaling through interactions with $\alpha_v\beta3$ integrin. Blocking ERK and TGF-β signaling overcomes EDIL3 induced angiogenesis and invasion. Using the orthotopic xenograft mouse model of HCC, we demonstrated that EDIL3 enhanced the tumorigenic, metastatic and angiogenesis potential of HCC *in vivo*.

Conclusions: EDIL3-mediated activation of TGF-β and ERK signaling could provide therapeutic implications for HCC.

Keywords: Hepatocellular carcinoma; Epithelial-mesenchymal transition; EDIL3; ERK; TGF-β; Tumour angiogenesis.

Received 7 January 2015; received in revised form 30 March 2015; accepted 4 May 2015; available online 14 May 2015

* Corresponding author. Address: Division of Cellular and Molecular Research, National Cancer Centre, Singapore. Tel.: +65 6436 8338; fax: +65 6226 3843.

E-mail address: cmrhkm@nccs.com.sg (K.M. Hui).

Abbreviations: HCC, hepatocellular carcinoma; EDIL3, EGF-like repeat and discoidin I-like domain-containing protein 3; DEL1, endothelial cell locus 1; IHC, immunohistochemistry; CTCs, circulating tumour cells; CFI, cancer-free interval; DMEM, Dulbecco's Modified Eagle's medium; HBV, hepatitis virus B; HCV, hepatitis virus C; RIPA, radioimmunoprecipitation; RT-PCR, Reverse Transcription-Polymerase Chain Reaction; ELISA, enzyme-linked immunosorbent assay; SCID, severe combined immunodeficiency; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labelling; MVD, microvessel density; BrdU, 5-Bromo-2′-deoxyuridine; IPA, Ingenuity Pathway Analysis.

Introduction

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer and one of the leading causes of cancer-related deaths worldwide [1]. Population-based studies have shown that the incidence rate continues to approximate the death rate of HCC, indicating that most patients who develop HCC die of the disease [1]. Some of the recognized risk factors associated with HCC include chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV), excessive alcohol intake, exposure to aflatoxin and, more recently added, chronic lifestyle diseases such as diabetes and obesity [2]. Despite recent advances in our understanding of the genetic landscape of HCC, the molecular mechanisms underlying hepatocarcinogenesis remain unclear and the prognosis for patients with advanced HCC remain dismal [3,4]. Early recurrence, metastasis and angiogenesis are the major obstacles to improving the clinical outcome of these patients [5,6].

The epithelial-mesenchymal transition (EMT) is a key step in cancer recurrence and metastasis by which epithelial cells lose their cell polarity and cell-cell adhesion, and gain migratory and invasive properties to become mesenchymal cells [7–9]. The EMT process enables cancerous cells to depart from the primary tumour, invading surrounding stromal tissue and be disseminated to distant organs. EMT has also been shown to confer efficient tumorigenicity to murine breast cancer cells by upregulating their expression of the proangiogenic factor VEGF-A and increasing tumour angiogenesis [10]. The expression of EMT markers, including vimentin, twist, ZEB1, ZEB2, snail, slug and E-cadherin, has been investigated in primary HCC tumours, adjacent non-tumoural liver tissues and circulating tumour cells

(CTCs) in HCC patients [11,12]. It has been reported that a decreased expression of E-cadherin in HCC patients significantly reduced the cancer-free interval (CFI) and induced a more aggressive phenotype [13]. Moreover, overexpression of Snail and Twist has been associated with the downregulation of E-cadherin and reduced overall survival [11]. Tissue microarray studies also demonstrated that overexpression of vimentin was significantly associated with HCC metastasis [14]. These observations demonstrated that EMT regulatory molecules may play critical roles in HCC progression.

To identify novel EMT regulatory molecules and investigate their correlation with angiogenesis, recurrence and metastasis in HCC, we have analysed and compared the gene expression profiles between samples of HCC patients with early recurrent disease and liver cancer cell lines with epithelial or mesenchymal phenotype [15,16] to identify potential novel EMT genes associated with the early recurrence of HCC.

Materials and methods

All the details of Materials and Methods are provided in Supplementary Materials and Methods (available online).

Results

EDIL3 is significantly upregulated in HCC samples of patients with early recurrent disease and poor survival

We have previously established a global gene expression profile database of histologically normal liver tissues and tumour tissues of HCC patients with early recurrent disease using Affymetrix Human Genome U133 plus 2.0 arrays [16]. To facilitate the identification of novel early recurrence-related genes associated with EMT in HCC, we have analysed the expression profiles of samples of HCC patients with early recurrence (“early recurrence” has been defined as recurrent disease detected within a two-year time duration after curative hepatic resection) in conjunction with the expression profiles of liver cancer cell lines with demonstrated epithelial or mesenchymal phenotype based on the expression of E-cadherin and vimentin [15,16]. A group of 23 genes which expression was modulated were shortlisted (Fig. 1A, Supplementary Table 1). Among these, the expression of EDIL3 was upregulated in samples of early HCC recurrence compared to histologically normal liver tissues. By qRT-PCR analysis, it was further demonstrated that EDIL3 expression was more markedly increased in samples of HCC patients with early recurrent disease compared to samples of HCC patients with non-recurrent disease (Fig. 1B). The patients’ clinicopathological features in HCC and survival univariate and multivariate analyses were shown in the Supplementary Table 2. Moreover, the expression of EDIL3 was shown to have a significant positive correlation with vimentin (VIM), a mesenchymal marker and negative correlated with E-cadherin (CDH1), an epithelial marker (Fig. 1C and D). The expression of EDIL3 was further studied in an independent cohort of 20 pairs of HCC tumour tissues (10 T-R and 10 T-NR) by IHC staining and EDIL3 protein expression was significantly increased in HCC tumour tissues (Fig. 1E and F). Since EDIL3 is known to be produced by endothelial cells, we also used the double-staining immunohistochemistry for the endothelial marker CD34 (brown staining) and EDIL3 (red staining). We observed that EDIL3 is also expressed in some intratumoural endothelial cells (Supplementary Fig. 1A). Interestingly, the results also indicated that EDIL3 produced by tumoural cells promote adjacent endothelial cell growth (Supplementary Fig. 1B). The correlation between EDIL3 and vimentin/E-Cadherin expression was also indicated by immunohistochemistry analysis (Supplementary Fig. 1C). When the median EDIL3 expression was calculated for all the fifty HCC samples studied by qRT-PCR and used as the cut-off for Fisher’s exact test and Kaplan-Meier analysis, it was demonstrated that high EDIL3 expression was significantly associated with a shorter overall survival (Fig. 1G). Consistent with EDIL3 being a secreted glycoprotein, EDIL3 protein expression was also shown to be significantly higher in the plasma of HCC patients compared to normal individuals by ELISA analysis. It is also shown that the EDIL3 level is significantly higher in the HCC patients with early recurrence than the HCC patients without early recurrence, suggesting EDIL3 can be a potential non-invasive diagnostic biomarker for HCC with early recurrence (Fig. 1H).

EDIL3 is a novel regulator of EMT in HCC

Previously, we established that epithelial liver cancer cells such as HepG2, Hep3B, HuH7 and PLC/PRF/5 had high CDH1 and low VIM expression. In comparison, liver cancer cells with a mesenchymal phenotype such as HLE, SK-HEP-1, SNU-449 and Mahlavu had low CDH1 and high VIM expression [17]. When the expression of EDIL3 was studied with the same panel of liver cancer cell lines by qRT-PCR and Western blotting, we observed that EDIL3 expression was significantly higher in liver cancer cells with a mesenchymal phenotype than in the cells with an epithelial phenotype (Fig. 2A and B). Similar observations could be made using independent published microarray data for liver cancer cell lines [15]. EDIL3 expression was significantly correlated with expression of the mesenchymal marker VIM and inversely correlated with the epithelial marker CDH1 (Supplementary Fig. 2A and B).

Next, epithelial HuH7 cells were stably transfected with either pLenti-EDIL3 or pLenti-control vector. The stable cells were tentatively designated as HuH7-EDIL3 or HuH7-control, respectively. The expression of EDIL3 in these cells was confirmed by qRT-PCR (Supplementary Fig. 2C). Compared to pLenti-control-transfected cells, the upregulation of EDIL3 was associated with dramatic morphological changes observed in the HuH7-EDIL3 cells: from an epithelial cobblestone phenotype to an elongated fibroblastic phenotype, which is indicative of EMT (Fig. 2C). The induction of EMT in the HuH7-EDIL3 cells was also associated with reduced E-cadherin and elevated vimentin expression (Fig. 2E and G). Similarly, HLE cells with a mesenchymal phenotype and high EDIL expression were used as the recipient cells for the transfection of EDIL3 shRNA. Following the silencing of EDIL3 (Supplementary Fig. 2D), striking morphological changes consistent with those of the mesenchymal-to-epithelial transition (MET) were observed (Fig. 2D). The upregulation of E-cadherin and reduced vimentin expression were also observed (Fig. 2F and H).

Effect of EDIL3 expression on HCC cell migration and invasion in vitro

EMT has been indicated as a key step in initiating cancer cell migration [17]. The migration potential of the HuH7-EDIL3 and HLE-shEDIL3 cells was studied using the wound healing assay. It was observed that stable overexpression of EDIL3 in the epithelial