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ZEB2 promotes vasculogenic mimicry by TGF-β1 induced epithelial-to-mesenchymal transition in hepatocellular carcinoma

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ABSTRACT

Aims: Zinc finger E-box binding homeobox 2 (ZEB2), an epithelial–mesenchymal transition (EMT) regulator, has been involved in invasion and metastasis of human tumor. Although EMT may be involved in vasculogenic mimicry (VM) formation, no reports describing the relation between ZEB2 and VM are available. We hypothesize that ZEB2 may promote VM formation in hepatocellular carcinoma (HCC).
Methods and results: Paraffin-embedded tumor tissue samples from 92 patients were immunostained with anti-ZEB2 antibody. We found that the ZEB2 nuclear expression was significantly associated with VM formation and metastasis. Patients with VM and ZEB2 nuclear expression had a shorter survival period than those without expression. In vitro, ZEB2 overexpression significantly enhanced cell motility, invasiveness, and VM formation of HepG2 cells. ZEB2 upregulation also increased VE-cadherin, Flt-1, and Flk-1 expression and activated MMPs. ZEB2 knockdown inhibited cell motility, invasiveness, and VM formation in Bel7402 cells. ZEB2 knockdown also decreased VE-cadherin, Flt-1, and Flk-1 expression and MMP activity. In addition, EMT in HepG2 cells was induced by TGF-β1 treatment, and the kinetics of expression of EMT markers and regulators were assessed by Western blot analysis. The expression of ZEB2 increased significantly, and VM formation was promoted.
Conclusion: ZEB2 can promote VM formation through the EMT pathway. Our findings may represent a novel therapeutic target in HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent primary malignancies of the liver worldwide, and both the incidence and mortality rates of HCC have been steadily increasing in recent years (Jemal et al., 2007; Yang and Roberts, 2010). Unfortunately, the long-term survival of patients with HCC remains unsatisfactory despite significant advances in surgical techniques and medical treatment (Hsu et al., 2006). This poor prognosis is primarily related to the high incidence of recurrence and metastasis of the disease (Tang et al., 2004; Yang et al., 2007). Adequate blood supply is the main cause of recurrence and metastasis. As a unique pattern of blood supply, vasculogenic mimicry (VM) describes the ability of some tumor cells, particularly aggressive ones, to form periodic acid-Schiff-positive and CD31-negative cells, express multiple endothelial markers, and resemble endothelial cell functions (Seftor et al., 2002; Zhang et al., 2007). VM is associated with the invasive and metastatic potential of tumor cells, as well as poor clinical outcomes (Maniotis et al., 1999; Sun et al., 2004).

Epithelial–mesenchymal transition (EMT) is a dynamic biological process characterized by epithelial cells' loss of epithelial characteristics and gain of properties typical of mesenchymal cells (Millimaggi et al., 2009). Epithelial tumor cells capable of VM imitate endothelial functions. They display some endothelial phenotypes of mesenchymal cells, which are similar to the EMT process. However, the cellular and molecular events between EMT mechanism and VM formation are not well understood. The regulators that contribute to EMT are assumed to be associated with VM formation. Previous studies have shown that Twist1 and ZEB1 (zinc finger E-box binding homeobox 1), as EMT inducers, are closely associated with tumor cell plasticity to VM patterns in HCC and colorectal carcinoma (CRC) (Liu et al., 2012; Sun et al., 2010).

Zinc finger E-box binding homeobox 2 (ZEB2) was originally identified in a transforming growth factor-β/bone morphogenetic protein (TGF-β/BMP) signaling pathway by its binding to the MH2 domain of receptor-activated Smads (Verschueren et al., 1999). ZEB2 has been

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thoroughly studied to bind the E-cadherin promoter and suppress the expression of this cell–cell adhesion molecule (Comijn et al., 2001; van Grunsven et al., 2003). Apart from E-cadherin, ZEB2 also directly represses the expression of the tight junction proteins, the desmosome proteins, and the gap junctions in a coordinated fashion (Vandewalle et al., 2005). ZEB2 is generally correlated to tumor metastasis, differentiation grade, and poor prognosis. Taken together, these data established ZEB2 as a critical regulator in promoting EMT and tumor progression. However, the relationship of ZEB2 and VM with HCC is currently unknown. In this study, we try to identify the potential contribution of ZEB2 to tumor VM formation and thus provide novel therapeutic strategies for HCC.

2. Materials and methods

2.1. Patient samples

Through the Tumor Tissue Bank of Tianjin Cancer Hospital, tissue specimens were obtained from 92 patients who underwent hepatectomy for HCC between 2005 and 2010. The diagnoses of these HCC samples were verified by pathologists. The use of these tissue samples was approved by the Ethical Committee of Tianjin Medical University, China.

2.2. Immunohistochemical staining and CD31/periodic acid Schiff double-staining

The assay was performed as previously described (Sun et al., 2010, 2011). The sections were incubated with rabbit ZEB2 polyclonal antibody (1:100, ab25837; Abcam, Cambridge, UK). The results were quantified according to the method described by Bittner et al. (Bittner et al., 2000).

2.3. Cell culture and treatment

The HCC cell lines used in this study were HepG2, Bel7402, PLC, and SMMC7221 (from the American Type Culture Collection, Rockville, MD). These cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen). For TGF-β1 treatment, HepG2 cells were seeded at 50% confluence in complete medium containing 10% fetal bovine serum. After 24 h, the cells were changed to the medium containing 1% fetal bovine serum, and TGF-β1 (Sigma) was added at a concentration of 10 ng/mL for further characterization.

2.4. Plasmids and transfection

The plasmids carrying ZEB2 and ZEB2 shRNA (or shZEB2) were purchased from GeneCopoeia (Guangzhou, China). The vectors were transfected into cells via percutaneous ethanol injection (Polysciences, Inc., Cat#23966).

2.5. Invasion and wound healing assay

Cell migration assay was performed using Transwell cell culture inserts (Invitrogen). The transfected cells were maintained for 48 h and allowed to migrate for another 24 h. The passed cells were stained with crystal violet solution, and their absorbance was determined at 595 nm. In wound healing assays, cell motility was assessed by measuring the movement of cells into a scarped. The speed of wound closure was monitored after 24 and 48 h by initially measuring the ratio of the distance of the wound at 0 h. Each experiment was performed in triplicate.

2.6. 3D cultures

The assay was performed as previously described (Liu et al., 2012; Sun et al., 2010, 2011).

2.7. Western blot analysis

All cell lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore). Blots were blocked and incubated with primary antibodies (ZEB2 1:500, E-cadherin 1:200, Vimentin 1:1000, VE-cadherin 1:500, Flt-1 1:200, Flk-1 1:200, Twist1 1:100, Slug 1:1000, and Snail 1:1000), followed by incubation with a secondary antibody (1:2000; Santa Cruz Biotechnology). Blots were developed using an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Pis-cataway, NJ). A monoclonal beta-actin antibody (1:200; Santa Cruz Biotechnology) was used for protein loading analyses.

2.8. Immunofluorescence staining

Cells were plated onto chamber slides and fixed in ice-cold methanol. The primary antibodies ZEB2, E-cadherin and Vimentin were used at 1:100 working solution. The fluorescein isothiocyanate and tetramethylrhodamine isothiocyanate-conjugated rabbit immunoglobulin G antibody (Santa Cruz Biotechnology) were used as labels for immunofluorescence assay. After immunolabeling, cells were washed, stained with DAPI (Sigma), mounted, and then viewed with fluorescent microscopy (Nikon, Japan).

2.9. Zymography assays

The assay was performed as previously described (Sun et al., 2010).

2.10. Statistical analysis

The correlation between ZEB2 expression and VM formation was analyzed using Spearman's rank test. The relationship between ZEB2 expression and clinicopathologic characteristics was analyzed using two-tailed Chi-square test method. Survival curves were estimated using Kaplan–Meier method. All data were evaluated with SPSS version 17 software (SPSS Inc.). Differences were considered significant at values of $P < 0.05$.

3. Results

3.1. Expression of ZEB2 was significantly associated with VM in HCC and its clinicopathological characteristics

VM was found in 17 out of 92 HCC samples (18%) by CD31/PAS double staining (Fig. 1A). According to VM presence, all samples were divided into two groups: VM-positive group ($n = 17$) and VM-negative group ($n = 75$). Of the 92 HCC samples, ZEB2 cytoplasm expression could be detected in 13 of the 17 (76%) samples in the VM-positive group and in 40 of the 75 (53%) samples in the VM-negative group. ZEB2 nuclear expression was detected in 7 of the 17 (41%) samples in the VM-positive group and in 14 of the 75 (19%) samples in the VM-negative group (Fig. 1B). The difference of ZEB2 cytoplasm expression in the VM-positive and VM-negative group was not significant, but the ZEB2 nuclear expression was significant difference between the VM-positive and the VM-negative groups ($P < 0.05$) (Table 1). As shown in Table 2, we also found the ZEB2 nuclear expression correlated with VM formation ($P = 0.046$, $r = 0.208$). A Kaplan–Meier survival analysis revealed that patients with VM and expression of ZEB2 (nuclear) had a shorter survival period than those without expression (Fig. 1C). We analyzed the relationship of ZEB2 and clinicopathological characteristics by SPSS, which showed that the expression of ZEB2 was not correlated with age, sex, tumor size, and histological types. However, significant correlation was observed between ZEB2 nuclear expression and pathological stage and metastasis of cancer (Table 1). Together, these results indicate that ZEB2 nuclear expression has a significantly associated with VM formation and metastasis.