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ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

Clinicopathological Significance of ZEB1 Protein in Patients with Hepatocellular Carcinoma

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ABSTRACT

Background. ZEB1, a member of the ZFH family of proteins (zinc-finger E-box binding homeobox), plays a central role in epithelial–mesenchymal transition (EMT) during carcinogenesis. In this study, we investigated the expression of ZEB1 in patients with hepatocellular carcinoma (HCC) and its clinical effects with underlying mechanisms.

Methods. Expression levels of ZEB1 were assessed by Western blot in 5 HCC cell lines and in paired cancerous and noncancerous tissues from 110 patients with HCC. Short-hairpin RNA (shRNA) interference for ZEB1 was performed in MHCC-97H cell line.

Results. ZEB1 protein was detected at a relatively high level in metastatic human HCC cell lines (MHCC-97L and MHCC-97H) when compared with that in nonmetastatic HCC cell lines (Hep3B, PLC and Huh-7). ZEB1 was expressed at high levels in 72 of 110 HCC patients (65.4%) and correlated with advanced TNM stage, tumor size >5 cm, intrahepatic metastasis, vascular invasion, and frequent early recurrence. The results of multivariate analysis revealed that ZEB1 high expression was a significant prognostic factor for poor overall and disease-free survivals. Silencing ZEB1 resulted in significant suppression of motility of MHCC-97H cell line, which was accompanied with increased expression of the epithelial marker E-cadherin and decreased expression of the mesenchymal markers N-cadherin and vimentin. Furthermore, silencing ZEB1 prevented the spread of intrahepatic metastasis and increased overall survival in mouse orthotopic tumor models.

Conclusions. This study shows that ZEB1 high expression was correlated with HCC malignant progression and subsequent poor patient survival by induction of EMT changes.

Hepatocellular carcinoma (HCC) is a major health problem worldwide, with an estimated incidence ranging between 500,000 and 1,000,000 new cases annually. It is the fifth most common cancer in the world, and the third most common cause of cancer-related death.[1,2] The disease is highly lethal because of its aggressive metastasis and the fact that it is often diagnosed at an advanced stage. Increasing evidence indicates that aberrant activation of the embryonic programme "epithelial–mesenchymal transition" (EMT) plays a key role in tumor cell invasion and metastasis. EMT allows detachment of cells from each other and increases cell mobility, both of which are necessary for tumor cell dissemination. A hallmark for EMT is the loss of the cell adhesion molecule E-cadherin. Several transcription factors have been described as key inducers of EMT, including members of the Snail superfamily (e.g. Snail1 and Snail2), the basic helix–loop–helix (bHLH) family [E47 (also known as TCF3) and TWIST] and the two zinc-finger E-box-binding homeobox (ZEB) factors (ZEB1 and ZEB2).[3,4]

Recent data indicate that ZEB1 has emerged as a key player in cancer progression.[5–12] Aberrant expression of ZEB1 in endometrial cancers, colorectal carcinomas and prostate cancer has been associated with aggressive

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disease, poor differentiation, development of metastases and poor clinical prognosis.[6,7,9,10] Up to now, no data are available regarding the role of ZEB1 in HCC. We thus conducted a study to evaluate expression of ZEB1 in patients with HCC and its clinical effects with underlying mechanisms.

MATERIALS AND METHODS

Cell Lines and Animals

Normal hepatocyte cell line HL-7702, nonmetastatic human HCC cell lines Hep3B, PLC, Huh-7 and PLC were purchased from the American Type Culture Collection (Manassas, VA), two metastatic human HCC cell lines MHCC-97L and MHCC-97H (low and high metastatic potential, respectively) were obtained from the Liver Cancer Institute, Fudan University, Shanghai, China.[13] All cells were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) and 1% penicillin and streptomycin in a 37°C incubator supplied with 5% CO_2.

Four- to 6-week-old male BALB/c nu/nu mice were supplied by Shanghai Centre of Experimental Animals, Chinese Academy of Science, kept in the local central animal facility of the Second Military Medical University, and housed in laminar-flow cabinets under specific pathogen-free conditions. All studies on mice were conducted in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animal.

Patient Samples

One hundred and ten HCC patients who underwent curative hepatic resection between March 2004 and June 2007 were recruited from Department of Special Treatment and Liver Transplantation, Eastern Hepatobiliary Surgery Hospital, Shanghai, China, contributing to 110 pairs of protein extracts from tumor tissues and adjacent nontumor tissues. The study was approved by the Ethics Committee of our hospital. Curative resection was defined as removal of all recognizable tumor with a clear microscopic margin. Specimens were obtained immediately after surgical resection. None of patients were treated by any preoperative therapy such as transcatheter arterial embolization, or percutaneous ethanol injection. There were 98 men and 12 women, ranging in age from 31 to 78 years, with a median age of 54 years. One hundred three HCC patients (93.6%) were positive for hepatitis B surface antigen. Tumor stage was defined according to American Joint Committee on Cancer/International Union Against Cancer tumor, node, metastasis (TNM) classification system.[14]

After discharge, patients were followed-up every one month by tumor marker (alfa-fetoprotein, AFP) analysis and ultrasound or computed tomography at least every three months at our outpatient clinic, especially during the first 2 years. Patients who developed recurrence were treated with repeat resection whenever possible, or by transcatheter arterial chemoembolization, percutaneous ethanol injection, or radiofrequency ablation as appropriate. According to point of recurrences time from the date of hepatectomy, recurrences were classified into early ($\leq$1 year) and late (>1 year) recurrences.[15]

Western Blot Analysis

Proteins from clinical specimens and HCC cell lines were extracted with lysis buffer (Beyotime, Shanghai, China), separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. The membrane was blocked with 5% nonfat dried milk in TBST (20 mM Tris–HCl, 150 mM NaCl, and 0.1% Tween 20, pH 7.5) for 2 h and incubated overnight with primary antibodies at a proper dilution at 4°C. After the wash with TBST buffer, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature and detected by enhanced chemiluminescence detection system (MultiScience Biotech, Shanghai, China). Antibodies against ZEB1, E-cadherin, N-cadherin, vimentin, and GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The intensity of the bands was quantified by densitometry and normalized to GAPDH.[16]

Establishment of Stable shRNA Transfectant

Three short-hairpin RNA (shRNA) sequences (sample 1, 2 and 3) targeting human ZEB1 and a sequence nonspecific to any known gene (as a control) were designed and individually cloned into pGCsil/U6/Purmycin shRNA expression vector (Stratagene, La Jolla, CA) according to manufacturer's instruction. Control shRNA vector and recombinant ZEB1-targeted shRNA plasmids were transfected into MHCC-97H cell line. Clone with the highest suppression of ZEB1 expression, carrying the sequence of sample 1 (sense: 5′- AACGTCACATGACATCACATAT ATGTGATGTCATGTGACGTT-3′; antisense: 5′- AACG TCACATGACATCACATATATGTGATGTCATGTGAC GTT-3′) was selected for cotransfected with lentiviral packaging vectors into 293T cells using lipofectamine 2000 (Invitrogen). After 48 h, lentivirus supernatant was harvested and concentrated. The virus titer was determined and calibrated in the 293T cell lines. The recombinant lentivirus was then transfected into the indicated cell lines

and stable clones were selected using 2.5 μg/ml of puromycin. MHCC-97H-shZEB1 and MHCC-97H-control represent cell lines stably transfected with recombinant shZEB1 vector and control shRNA vector, respectively.

Wound Healing Assay

Cells (2×10^4) were implanted onto a 24-well plate. When the cells grown to 80% confluence, an acellular area was created by a 200 μl pipette tube (time 0), and the speed of wound closure was monitored after 24 h under 100× magnification.[17] At least three independent experiments were performed.

Migration Assay

Cell migration assays were performed using the 8 μm BD Falcon cell culture Chambers (BD Biosciences). Cells (2×10^4) were suspended in 0.5 ml serum-free DMEM, then seeded into the upper compartments of each chamber, and the lower compartments were filled with 1 ml DMEM supplemented with 10% FBS. After 24 h incubation at 37°C in 5% CO_2, nonmigrating cells were removed by scrubbing from the upper surface of the membrane. Cells on the reverse side were stained with 0.1% crystal violet, and the numbers of cells were counted in five microscopic fields (×200).[18] At least three independent experiments were performed.

Orthotopic Tumor Models

Orthotopic tumors were established as described previously.[13] For the in vivo model, MHCC-97H-shZEB1 and MHCC-97H-control ($n = 16$ in each group) were implanted. On 35 days after tumor cell inoculation, 16 animals ($n = 8$ in each group) were sacrificed, and tumor bearing liver lobes were fixed in formalin (4%). To examine the intrahepatic metastases, 100 sequential sections (4 μm) were cut and stained with H&E as described previously.[13] The remaining 16 mice were left for assessing survival.

Statistical Analysis

Categorical data were compared using chi-square or Fisher exact tests. Patient survival was calculated using the Kaplan-Meier method and compared by the log rank test. Multivariate analysis of prognostic factors for survival was performed by a Cox stepwise regression model. All statistical analyses were performed with SPSS statistical software version 9.0 for Windows (SPSS, Chicago, IL). $P < 0.05$ was considered statistically significant.

RESULTS

ZEB1 Expression in Different Cell Lines

The index of ZEB1 expression in MHCC-97L, MHCC-97H, Hep3B, PLC and Huh-7 was 0.64, 0.68, 0.33, 0.39 and 0.54, respectively. ZEB1 protein was detected at a relatively high level in metastatic HCC cell line MHCC-97L and MHCC-97H when compared with that in Hep3B, PLC and Huh-7, and detected at the highest level in MHCC-97H with high metastatic potential. Normal hepatocyte cell line HL-7702 showed weak protein expression with an index of 0.28 (Fig. 1a).

ZEB1 Expression in HCC

The expression level of ZEB1 protein was higher in tumors than that in the corresponding nonmalignant tissues (Fig. 1b). Overall, the mean expression index of ZEB1 was 0.53 ± 0.04 for tumor samples and 0.26 ± 0.03 for the corresponding nonmalignant tissues ($P < 0.01$). The ZEB1 protein level of nonmalignant livers rarely exceeded a ratio of 0.3. Therefore, a ZEB1-to-GAPDH ratio >0.3 was defined as ZEB1 protein high expression.

High expression of ZEB1 was detected in 65.4% (72/110) of HCC tissues. To clarify the correlation between the ZEB1 expression and the invasiveness of HCC, the clinicopathological variables between the ZEB1 expression patterns were compared (Table 1). High expression of ZEB1 protein was significantly correlated with advanced TNM stage ($P = 0.01$), tumor size >5 cm ($P = 0.027$), intrahepatic metastasis ($P = 0.031$), vascular invasion ($P = 0.006$), and frequent early recurrence ($P = 0.013$). There was no significant correlation between ZEB1 expression and sex, age, cirrhosis, tumor capsule formation, AFP level or hepatitis B surface antigen.

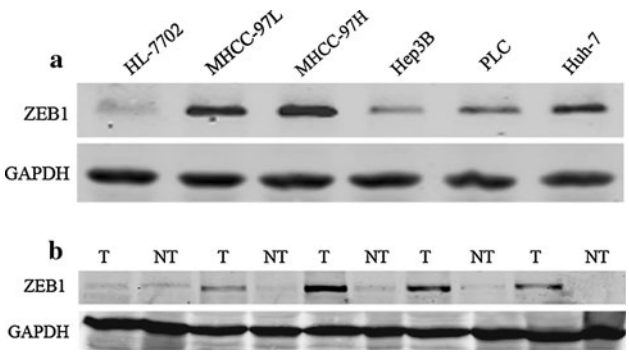

FIG. 1 Western blot analysis of ZEB1 protein expression. **a** Different cell lines. **b** Tumor tissues (*T*) and nontumor tissues (*NT*; surrounding liver tissues)

TABLE 1 Clinicopathologic correlation with ZEB1 expression in HCC

Characteristic	Total no.	ZEB1 low expression	ZEB1 high expression	*P* value
Age (years)				
≤60	73	28	45	0.238
>60	37	10	27	
Gender				
Male	98	34	64	0.925
Female	12	4	8	
Hepatitis B surface antigen				
Negative	7	2	5	0.731
Positive	103	36	67	
Serum AFP (ng/ml)				
≤200	79	31	48	0.098
>200	31	7	24	
TNM stage				
I	12	8	4	0.010
II	30	13	17	
III–IV	68	17	51	
Tumor size (cm)				
≤5	37	18	19	0.027
>5	73	20	53	
Intrahepatic metastasis				
Absent	82	33	49	0.031
Present	28	5	23	
Cirrhosis				
Absent	52	21	31	0.223
Present	58	17	41	
Tumor capsule formation				
Absent	66	19	47	0.12
Present	44	19	25	
Vascular invasion				
Absent	47	23	24	0.006
Present	63	15	48	
Early recurrence				
Yes	34	6	28	0.013
No	76	32	44	

ZEB1 Expression Correlated with Poor Survival

Kaplan-Meier analysis showed that ZEB1 high expression was significantly associated with poor overall survival (log rank = 6.570, $P = 0.01$, Fig. 2a) and disease-free survival (log rank = 4.174, $P = 0.037$, Fig. 2b) in HCC patients.

Multivariate analysis identified two factors (ZEB1 high expression and vascular invasion) significantly influencing the overall survival rate, and three factors (ZEB1 high expression, advanced TNM stage, and vascular invasion) significantly influencing the disease-free survival rate (Table 2). ZEB1 high expression was confirmed to be an independent favorable factor for overall and disease-free survival: the risk ratios with 95% confidence intervals and the *P* values were 2.222, 1.097–4.503, $P = 0.027$, and 1.814, 1.002–3.284, $P = 0.048$, respectively.

Effects of ZEB1 Silencing on Cell Motility

To investigate the effect of ZEB1 suppression on cell motility, two common methods including wound healing assay and migration assay were performed on MHCC-97H-control and MHCC-97H-shZEB1 cells. Wound healing assay showed that the wound almost recovered at 24 h after wounding in MHCC-97H-control while only slightly improved in MHCC-97H-shZEB1 (Fig. 3a). Migration assay showed that after a 24-h incubation, the number of migrated MHCC-97H-shZEB1 cells was significantly less than that of migrated MHCC-97H-control ($256.33.33 \pm 34.4$ versus 491.86 ± 34.5, $P < 0.01$) (Fig. 3b). The above results demonstrated that down-regulation of ZEB1 gene suppressed the motility of metastatic HCC cells.

Effects of ZEB1 Silencing on E-cadherin and Vimentin Expression

To explore the potential mechanisms of reduced motility in HCC cells with down-regulation of ZEB1, we examined the expression of epithelial and mesenchymal markers. As shown in Fig. 4, increased epithelial marker E-cadherin protein, and decreased mesenchymal markers N-cadherin and vimentin protein, were observed in the MHCC-97H-shZEB1 cells.

Effect of ZEB1 Silencing on Orthotopic Tumor Metastasis

In the in vivo animal model, tumors were removed 5 weeks after the implantation, and the sections stained with H&E. Liver tumors in MHCC-97H-control group showed an infiltrative growth pattern with frequently intrahepatic metastases. On the contrary, liver tumor in MHCC-97H-shZEB1 group showed an expansive growth pattern with rare intrahepatic metastases. The median survival time was 68 days in the MHCC-97H-shZEB1 compared with 53 days in the MHCC-97H-control ($P < 0.01$).

ZEB1 and E-cadherin Expression in HCC

To further assess the possible correlation of ZEB1 expression with E-cadherin, E-cadherin protein expression was analyzed in 10 HCC specimens with ZEB1 high expression and 10 HCC specimens with ZEB1 low

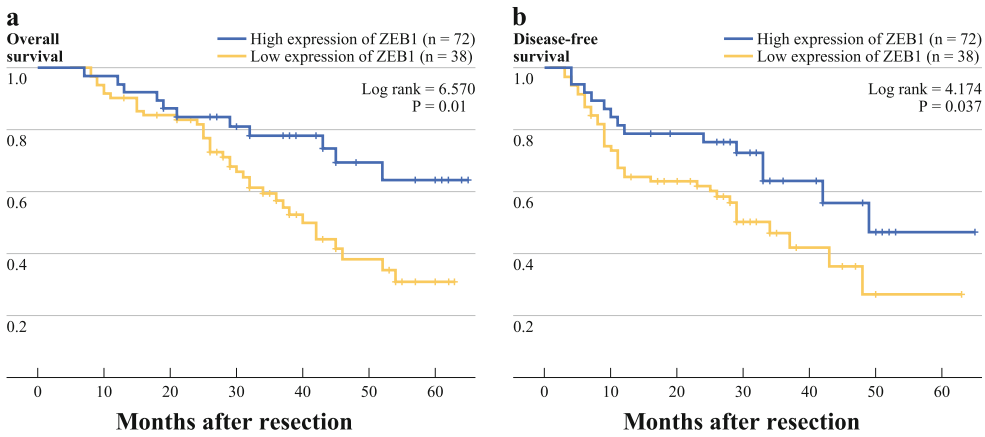

FIG. 2 Impact of ZEB1 expression on patient survival as evaluated by the Kaplan-Meier method. **a** Overall survival rate. **b** Disease-free survival rate

TABLE 2 Multivariate analysis

Characteristic	Risk ratio	95% confidence interval	*P* value
Overall survival			
ZEB1 high expression	2.222	1.097–4.503	0.027
Vascular invasion	2.325	1.256–4.305	0.007
Disease-free survival			
ZEB1 high expression	1.814	1.002–3.284	0.048
TNM stage (III–IV)	1.826	0.976–3.153	0.046
Vascular invasion	1.873	1.067–3.289	0.029

expression. We found the level of E-cadherin in ZEB1 high expression group was significantly lower than that of ZEB1 low expression group (0.28 ± 0.03 versus 0.36 ± 0.02, $P < 0.01$). The data suggested that ZEB1 expression negatively correlated with E-cadherin expression in HCC.

DISCUSSION

ZEB1 (also known as AREB6, ZFHEP, ZFHX1A, BZP, NIL-2-A, DeltaEF1) is a nonreceptor transcription factor analogous to the Drosophila ZFH-1 protein, and contains a conserved central homeobox region and two zinc-finger domains at the N- and C-terminal regions, by which it binds E-box-like sequences (CACCTG) on DNA.[19] ZEB1 has been implicated in multiple processes during development such as lymphopoiesis, skeletal patterning, chondrogenesis, neurogenesis, neural crest cell development.[20–23] More recently, compelling evidence has accumulated indicating that ZEB1 plays a central role in EMT during carcinogenesis.[24] Aberrantly expressed ZEB1 in endometrial cancers, colorectal carcinomas, and prostate cancer has been associated with aggressive disease, poor differentiation, development of metastases, and poor clinical prognosis.[6,7,9,10] However, the role of ZEB1 in HCC has yet to be elucidated.

In a mouse xenograft model of colon cancer, expression of ZEB1 promotes metastasis of tumor cells.[8] Human HCC cell lines MHCC-97H and MHCC-97L are from the same parent cell line with the same genetic background but with different metastatic potential.[13] Orthotopic implantation of MHCC-97H cells into nude mice results in 100% of lung metastasis, whereas MHCC-97L exhibits 40% of lung metastasis.[13] Our data showed that ZEB1 protein was higher expressed in both MHCC-97L and MHCC-97H, but not in nonmetastatic HCC cell lines Hep3B, PLC and Huh-7. Higher expression of ZEB1 protein in metastatic HCC cells suggested that ZEB1 may be a metastasis-associated oncogene, and participate in the process of metastases in HCC.

We found that the expression levels of ZEB1 protein were higher in tumors than that in the corresponding nonmalignant tissues. In addition, multivariate analysis showed ZEB1 high expression was an independent favorable factor for overall and disease-free survival, suggesting that ZEB1 can be used to predict patient outcomes.

The postoperative recurrence of HCC remains the major cause of death and the main obstacle to long-term survival.[25] According to point of recurrences time from the date of hepatectomy, recurrences were classified into early ($\leq$1 year) and late (>1 year) recurrences. The prognosis of patients with early recurrent tumors was found to be worse than that of patients with late recurrence despite a similar treatment regimen.[15] Early recurrence after resection of HCC is likely to be associated with aggressive tumor pathological factors such as high tumor grade, vascular invasion, and microsatellite lesions.[15,26] In the current study, the incidence of early recurrence in patients with ZEB1 high expression was significantly higher than that in patients with ZEB1 low expression. We also found that the

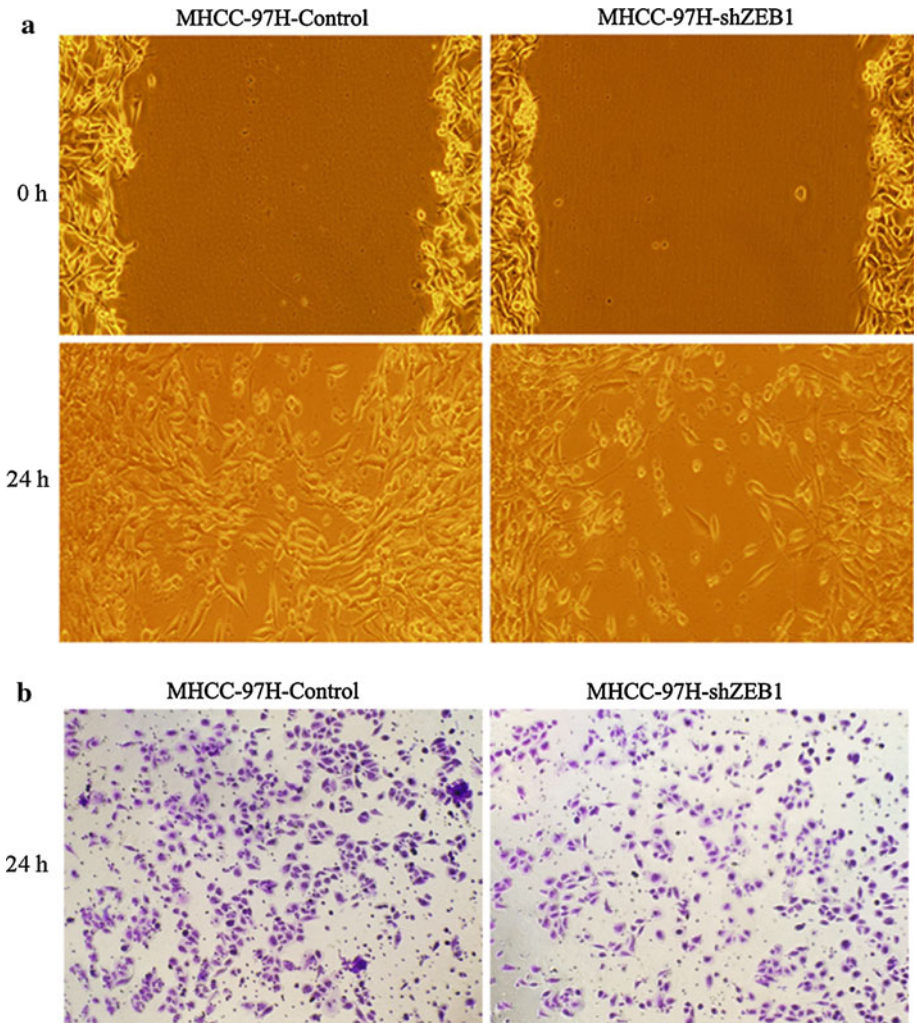

FIG. 3 Cell motility assays of MHCC-97H-control and MHCC-97H-shZEB1 cells. **a** Wound healing assay after 24 h. **b** Migration assay after 24 h

high expression of ZEB1 protein in HCC tissues was significantly correlated with advanced TNM stage, intrahepatic metastasis, tumor size >5 cm, and vascular invasion, indicating the possible role of ZEB1 in promoting HCC malignant progression, thereby leading to early recurrence and poor outcome, despite curative surgical resection.

To further confirm the role of ZEB1 in HCC malignant progression, we employed shRNA technology to suppress the expression of ZEB1 gene in a metastatic HCC cell line MHCC-97H with high expression of ZEB1 protein. In vitro assays showed that suppression of ZEB1 expression significantly reduced motility of MHCC-97H cell line. In vivo orthotopic tumorigenesis model also supported that inhibition of ZEB1 expression prevented the spread of intrahepatic metastasis and increased overall survival of model mice. These results were consistent with the clinical significance of ZEB1 in human HCC samples.

The loss of E-cadherin, a cell adhesion molecule, is considered a hallmark of EMT.[4] ZEB1, like other EMT-inducing transcription factors such as Twist, Snail, Slug, and SIP, binds DNA using similar E-box sequence motifs, repressing E-cadherin.[5] After ZEB1 expression was down-regulated, we found protein levels of the mesenchymal markers N-cadherin and vimentin were down-regulated, whereas expression of E-cadherin was enhanced. Furthermore, it was found that ZEB1 expression negatively correlated with E-cadherin expression in HCC samples. Taken together, these results indicated that ZEB1 might lead to the increased invasiveness of HCC cells by induction of EMT changes.

In summary, ZEB1 high expression was correlated with HCC malignant progression and subsequent poor patient survival by induction of EMT changes. ZEB1 might be a promising novel therapeutic target in human HCC.

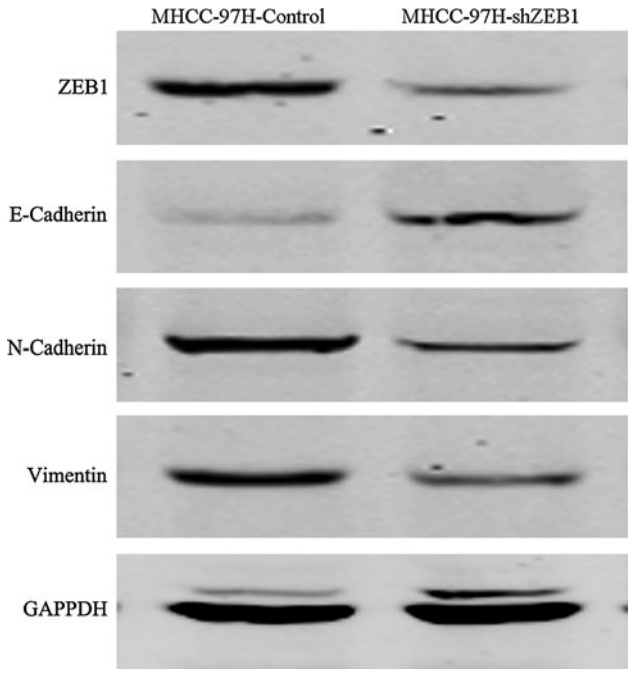

FIG. 4 Effects of ZEB1 silencing on E-cadherin and vimentin expression. After ZEB1 expression was down-regulated, protein levels of the mesenchymal markers N-cadherin and vimentin were down-regulated, whereas expression of E-cadherin was enhanced

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