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Levels of HBx, VEGF, and CEACAM1 in HBV-related hepatocellular carcinoma and their correlation with cancer prognosis

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Abstract. – OBJECTIVE: Hepatitis B virus X protein (HBx), vascular endothelial growth factor (VEGF) and carcinoembryonic antigen related cell adhesion molecule 1 (CEACAM1), are related to HBV associated hepatocellular carcinoma (HCC). This study recruited HCC patients and employed the SMMC-7721 and L02 liver cell lines, to analyze the expression levels of HBx, VEGF and CEACAM1 in liver cancer and their correlation with the cancer prognosis.

PATIENTS AND METHODS: HBV-related HCC patients were recruited from our hospital. Immunohistochemistry (IHC) and Western blotting assay were used to detect the expression of HBx, VEGF and CEACAM1 in liver tissues. Multi-variant analysis and the correlation analysis between HBx, VEGF, CEACAM1 expression and clinical/pathological features of HCC were performed by using the Cox regression analysis.

RESULTS: In HBV-related HCC tissues, positive expression rates of HBx, CEACAM1, and VEGF, were 80%, 50%, and 65%, respectively. In HBx-positive group, positive rate for CEACAM1 and VEGF were 56.25% and 75%, while in HBx-negative group such figures were 75% and 25% ($p<0.05$). HCC cells had lower expression of CEACAM1 and higher VEGF levels compared to normal hepatocytes. Those HCC cells transfected with HBx had even lower CEACAM1 and higher VEGF levels compared to un-transfected cells. HBx was negatively correlated with CEACAM1 and positively correlated with VEGF. Expressions of these three factors were all independent risk factors as they were correlated with lesion size, venous infiltration, metastasis, and capsule.

CONCLUSIONS: HBx, VEGF and CEACAM1 were widely expressed in HBV-related HCC. HBx may facilitate occurrence and progression of HBV-related HCC via down-regulating CEACAM1 and up-regulating VEGF.

Key Words:
HBV infection, Hepatocellular carcinoma, VEGF, CEACAM1.

Introduction

Hepatocellular carcinoma (HCC) is a common malignant tumor in clinics. Hepatitis B virus (HBV) is the major reason for HCC in China. Hepatitis B X protein (HBx) has been demonstrated to be closely related to HBV-related HCC[1]. In China, more than 80% HCC patients were HBV carriers. Once enter into hepatocytes of the host, HBV leads to instability of genome to affect cell proliferation and growth[2]. Tumor markers play an important role in cancer diagnosis. Carcinoembryonic antigen (CEA) was up-regulated in certain tumors[3-5]. There are 29 members in CEA family, in which carcinoembryonic antigen related cell adhesion molecule 1 (CEACAM1) was related with adhesion proteins[6]. Cruz et al[7] suggested the potency of CEACAM6 as tumor suppressor and the critical role in the progression of HBV-related HCC. Vascular endothelial growth factor (VEGF) can regulate angiogenesis of solid tumors, and induce the proliferation and migration of endothelial cells[8,9]. This study quantified the level of HBx, VEGF, and CEACAM1 to investigate the correlation among these three factors, and to elucidate their correlations with HBV-related HCC.

Patients and Methods

Patients

A total of 40 HBV-related HCC patients who received surgeries in Henan Province People's Hospital from January 2015 to January 2016 were recruited as the study group. All patients were confirmed as HCC without radio- or chemo-therapy before the surgery. The typical lesion was extracted during the surgery. Those with positive expression of serum hepatitis B surface antigen (HbsAg) or HBV covalently closed circular DNA

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(cccDNA) were identified as HBV-related HCC tissues. Among all patients, there were 20 males and 20 females aging between 31 and 81 years old (average = 51.23 ± 2.63 years). All patients had no significant difference regarding the sex or age (p>0.05). After resection, 100 mg samples were frozen at -80°C for further experiments. The study protocol was approved by the Research Ethics Committee of Henan Province People's Hospital, and all patients gave their informed consent before study commencement.

Cell Model

Human liver cancer SMMC-7721 cell and normal hepatocyte L02 cell lines were generous gifts for Huabei Pharma (Shanghai, China).

Reagents

Rabbit anti-human HBx, VEGF and CECAM1 polyclonal antibody, SP assay kit, DAB kit, Lysine, citric acid antigen retrieval buffer, xylene, absolute ethanol, paraffin and hematoxylin were purchased from Sangon Bio. Ltd. (Shanghai, China). Horse radish peroxidase (HRP)-conjugated rabbit anti-mouse secondary antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Dulbecco's Modified Eagle Medium (DMEM) culture medium, penicillin-streptomycin, and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA).

Equipment

Ultrapure workstation (Formal 205, Manteca, CA, USA), tissue embedding apparatus (Sakura, Japan), dehydration equipment (Sakura, Japan), microtome (Leica, Germany), drying chamber (Shengxin Instrument, Zhengzhou, China), CO_2 incubator (Sanyo, Moriguchi, Osaka, Japan); Micro-centrifuge (Beckman Coulter Inc., Brea, CA, USA), -80°C fridge (Sanyo, Moriguchi, Osaka, Japan) and Computer image analysis system (Hewlett Packard, Palo Alto, CA, USA) were used in this study.

Immunohistochemistry (IHC) staining

Tissue samples were fixed in formaldehyde, and dehydrated, embedded in paraffin. Tissues blocks were then sectioned by a microtome. Tissue slices were de-waxed, dehydrated, processed in antigen retrieval, and incubated in 3% hydrogen peroxide for 10 min. Blocking was performed using goat serum. Primary antibody (50 µl) was added for 1 h incubation at room temperature, followed by the addition of secondary antibody. Peroxidase (50 µl) was then added after 10 min for developing the color. After quenching, hematoxylin was used to counter-stain tissue slices. After differentiation by HCl-ethanol, tissue slices were dehydrated, mounted, and observed in an inverted microscope.

Cell Culture

Human liver cancer SMMC-7221 cell line and normal hepatocyte L02 cell line were incubated in RPMI1640 medium in a humidified chamber with 5% CO_2 at 37°C.

Cell Transfection

Human liver cancer SMMC-7221 cell suspensions were incubated in a humidified chamber with 5% CO_2 at 37°C. Tumor cells were then inoculated into culture dish at optimal density for overnight incubation. When reaching 60% confluence, lipofectamine 2000 was used to transfect HBx plasmid into to transfect normal cell with purchased HBx plasmids. After 4-6 h culture medium was changed for 48 h incubation. Un-transfected SMMC-7721 or L02 normal hepatocyte cell lines were used as control groups.

Western Blotting

Proteins (40 µg per well) were separated in 8% sodium dodecylsulphate sulphate-olyacrylamide gel electrophoresis (SDS-PAGE) following protein lysis. Next, separated proteins were transferred to a membrane, which was blocked at room temperature for 1 h. After discarding the blocking buffer, primary antibody diluent (1:200, 1:500 for β-actin) was added for 30 min incubation at room temperature, followed by 4°C overnight incubation. The membrane was washed in tris buffered saline-tween TBS-T buffer, and was incubated with secondary diluent (1:2000) for 1 h. After rinsing in phosphate buffer solution-tween 20 (TBS-T) buffer, chromogenic substrates A and B were sequentially added (2 ml each) for developing the membrane, which was scanned and analyzed for optical density using Quantity One software (Hercules, CA, USA).

Judging Criteria

Using IHC staining slides with known expression of target indexes as the positive control, we also recruited negative controls using PBS buffer instead of primary antibody diluents. The positive expression of HBx, VEGF and CEACAM1 was identified as previously recorded[9]: those with no nuclear staining, and yellow-brown granules in

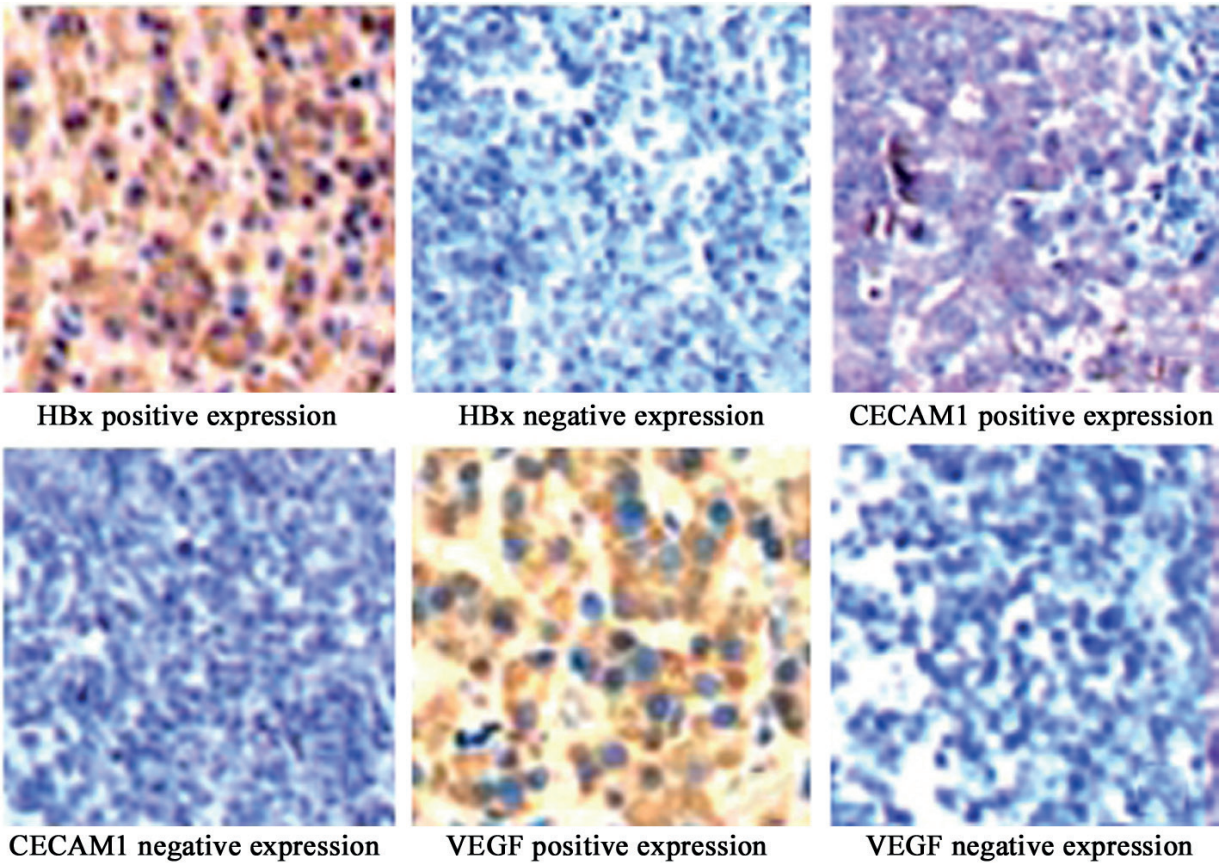

Figure 1. Expression of HBx, VEGF and CEACAM1 in HBV-related HCC tissues by immunohistochemistry (IHC) staining (×200). HBx: Hepatitis B virus X protein, VEGF: vascular endothelial growth factor, CEACAM1: carcinoembryonic antigen related cell adhesion molecule 1, HCC: hepatocellular carcinoma.

membrane or cytoplasm. Based on the percentage of positive cells, the whole tissue sample can be classified as negative (-), with less than 10% positive cells; weak positive (+), with 11-25% positive cells; positive (++), having 26-50% positive cells; strong positive (+++), with more than 50% positive cells.

Statistical Analysis

SPSS17.0 statistical software (SPSS Inc., Chicago, IL, USA) was used to process all experimental data, which were presented as mean ± standard deviation (SD). Enumeration data and measurement data were compared by X^2 test and Student *t*-test, respectively. The multi-variant analysis and the correlation analysis between HBx, VEGF, CEACAM1 expression, and clinical features, were performed by using the Cox regression analysis. Statistical significance was defined when $p<0.05$.

Results

HBx, VEGF and CEACAM1 Expression in Patient Tissues

We tested the expression levels of HBx, VEGF, and CEACAM1 in tumor tissues from all included HBV-related HCC patients. Results showed 32 (80%), 20 (50%) and 26 (65%) cases with positive expression of HBx, CEACAM1, and VEGF, respectively. In HBx-positive sub-group, there were 18 patients with CEACAM1-positive expression (56.25%). Such ratio was only 25% (2 out of 8) in HBx-negative patients, with significant difference compared to HBx-positive group ($p<0.05$). In HBx-positive group, there were 24 patients with VEGF-positive expression, occupying 75%, while in HBx-negative expression group, there were only 2 VEGF-positive individuals (25%), with significant difference compared to HBx-positive group (Table I and Figure 1).

Table I. HBx, VEGF and CEACAM1 expression in liver tissues of patients.

Index	HBx-positive group (32/40, 80%)	HBx-negative group (8/40, 20%)
CEACAM1		
-	43.75% (14/32)	75 (6/8)
+~+++	56.25% (18/32)*	25 (2/8)
VEGF		
-	25% (8/32)	75% (6/8)
+~+++	75% (24/32)*	25% (2/8)

Note: *, $p<0.05$ compared to HBx-negative group. HBx: Hepatitis B virus X protein, VEGF: vascular endothelial growth factor, CEACAM1: carcinoembryonic antigen related cell adhesion molecule 1, HCC: hepatocellular carcinoma.

Correlation Among HBx, VEGF and CEACAM1

There was a negative correlation between HBx and CEACAM1 levels (r=-0.31, $p<0.05$), and a positive correlation between HBx and VEGF level (r=0.552, $p<0.01$).

HBx, VEGF and CEACAM1 Expression in HBx-Transfected Cells

Western blotting was employed to detect HBx, VEGF, and CEACAM1 levels in HCC cells and normal liver cells, or HCC cells transfected with HBx found significant elevation of HBx expression after transfection. CEACAM1 level was highest in HBx-transfected cancer cells, followed by HCC cells and normal hepatocytes. Whilst VEGF showed the opposite pattern, as its level was gradually decreased in HBx-transfected HCC cells, HCC cells, and normal hepatocytes ($p<0.05$, Table II, Figure 2).

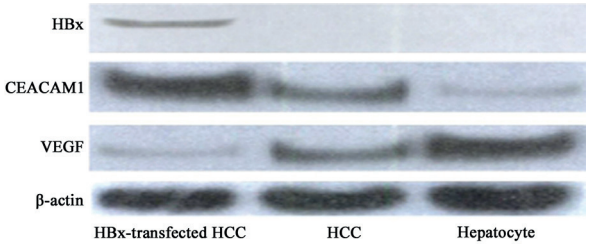

Figure 2. Expression of HBx, VEGF and CEACAM1. HBx: Hepatitis B virus X protein, VEGF: vascular endothelial growth factor, CEACAM1: carcinoembryonic antigen related cell adhesion molecule 1.

Correlation Between Expression of HBx, VEGF, CEACAM1 and Clinical Features

We analyzed the expression level of HBx, VEGF and CEACAM1 in cancer tissues with clinical or pathological features including patient sex, age, tumor size, venous infiltration, intra-/extra-hepatic metastasis, capsule, liver cirrhosis and alpha-fetoprotein (AFP). Results showed correlation between those expression levels with tumor size, venous infiltration, intra-/extra-metastasis and capsules. For those with tumor size larger than 5 cm, accompanying with venous infiltration, intra-/extra-hepatic metastasis, and without capsules, expression levels of HBx and VEGF were significantly elevated, while CEACAM1 expression level was down-regulated ($p<0.05$). No correlation has been found between expression levels of HBx, VEGF and CEACAM1 and patient sex, age, liver cirrhosis or AFP ($p>0.05$, Table III).

Table II. Expression of HBx, VEGF and CEACAM1.

HBx	CEACAM1	VEGF	
HBx-transfected HCC cells	$0.18 \pm 0.49^{*\#}$	$0.10 \pm 0.03^{*\#}$	$0.64 \pm 0.47^{*\#}$
Untransfected HCC cells	-	$0.22 \pm 0.21^{\#}$	$0.32 \pm 0.29^{\#}$
Normal hepatocytes	-	0.62 ± 0.51	0.09 ± 0.11

Note: *, $p<0.05$ compared to HCC cells; #, $p<0.05$ compared to normal hepatocytes. HBx: Hepatitis B virus X protein, VEGF: vascular endothelial growth factor, CEACAM1: carcinoembryonic antigen related cell adhesion molecule 1, HCC: hepatocellular carcinoma.

Table III. Correlation between VEGF/CEACAM1 expression and clinical feature of HCC.

Factor	N.	HBx (+)	p-value	VEGF (+)	*p*-value	CEACAM1 (+)	*p*-value
1. Sex							
Male	20	16		14		11	
Female	20	16	>0.05	12	>0.05	9	>0.05
2. Age (years)							
≤60	25	20		12		12	
>60	15	12	>0.05	14	>0.05	8	>0.05
3. Tumor size							
≤5 cm	22	15		8		5	
>5 cm	18	17	<0.05	18	<0.05	15	<0.05
4. Venous infiltration							
Yes	22	22		18		5	
No	18	10	<0.05	8	<0.05	15	<0.05
5. Metastasis							
Yes	24	23		19		6	
No	16	9	<0.05	7	<0.05	14	<0.05
6. Capsule							
Yes	12	5		3		9	
No	28	27	<0.05	23	<0.05	11	<0.05
7. Liver cirrhosis							
Yes	21	17		14		11	
No	19	15	>0.05	12	>0.05	9	>0.05
8. AFP							
<20 ng/ml	16	11		12		9	
>20 ng/ml	24	19	>0.05	14	>0.05	11	>0.05

HBx: Hepatitis B virus X protein, VEGF: vascular endothelial growth factor, CEACAM1: carcinoembryonic antigen related cell adhesion molecule 1, HCC: hepatocellular carcinoma, AFP: alpha-fetoprotein.

Multi-variant Analysis Between HBx, VEGF, CEACAM1 Expression and Clinical Features

Logistic multi-variant analysis was further performed for tumor size, venous infiltration, intra-/extra-hepatic metastasis and capsule, all of which were factors in single-variant analysis. Results showed those as independent risk factors (Table IV).

Discussion

HBV infection is a risk factor inducing HCC, with its protein products in the form of HBx[10]. A previous study found certain trans-activating function of HBx, which was classified as transcriptional activation factor, and can exert important roles on intracellular signal transduction within the body, affecting gene transcription, expression and cell division or proliferation, thus exerting critical function in occurrence and progression of HCC[11]. As an important family member of CEA, CEACAM1 is a trans-membrane glycoprotein, which is an important domain of immune super-family[12]. Wang et al[13] indicated that CEACAM1 could exert tumor suppressor function and had down-regulation in prostate cancer, renal cell carcinoma and liver cancer. Neo-angiogenesis is of critical importance for occurrence and progression of tumors, as

Table IV. Multi-variant analysis between HBx, VEGF, CEACAM1 expression and clinical features.

Index	Regression coefficient	*p*-value	Relative risk
Tumor size	0.801	0.004	2.228
Venous infiltration	1.275	0.001	2.852
Metastasis	1.018	0.001	2.812
Capsule	1.004	0.001	2.730

HBx: Hepatitis B virus X protein, VEGF: vascular endothelial growth factor, CEACAM1: carcinoembryonic antigen related cell adhesion molecule 1, HCC: hepatocellular carcinoma.

it can provide sufficient oxygen and nutrients for unlimited growth of malignant tumors. During the angiogenesis, both VEGF and basic fibroblast growth factor (bFGF) are involved, while VEGF also affected vessel formation in addition to angiogenesis[14,15]. In this study, HBV-related HCC patients were recruited for testing expression levels of HBx, VEGF, and CEACAM1 in liver tissues. Results showed 80% with HBx positive expression and 50% with CEACAM1 positive expression. In HBx-positive sub-group, the ratio of CEACAM1-positive was 56.25%, while in HBx-negative cohorts such ratio was 75%. The positive ratio of VEGF was 75% and 25% for HBx-positive and HBx-negative expression populations, respectively. These results showed the up-regulation of VEGF and down-regulation of CEACAM1 in HBx-positive HBV-related HCC patients. In order to further study the correlation among three factors at molecular level, human liver cancer SMMC-7721 cells were employed for HBx-transfection followed by expressional assay of HBx, VEGF, CEACAM1, in parallel with un-transfected liver cancer cells, or normal hepatocytes. We found significant elevation of HBx expression in liver cancer cells after HBx transfection, and gradually increased CEACAM1 levels in HBx-transfected liver cancer cells, un-transfected liver cancer cells and normal hepatocytes, and decreased VEGF levels in these cells. Further analysis revealed negative correlation between HBx and CEACAM1, and positive relationship between HBx and VEGF. These results indicated that over-expression of HBx could inhibit CEACAM1 expression via a series of signal transduction pathway. A previous study[16] indicated the possible mechanism of HBx-related HCC occurrence and progression via activating Notch signal pathway. Xu et al[17] suggested that HBx could decrease the level of multiple tumor suppressor genes, thus activating the uncontrolled tumor proliferation. Such model was further discussed by Paloma et al[18] who found that HBx could induce the alternation of angiotensin-2, thus inducing neo-angiogenesis of tumors, and facilitate liver cell invasion and metastasis. This work further analyzed the correlation between expressions of HBx, VEGF, and CEACAM1 in HBV-related HCC and clinical/pathological features. Results found no correlations between the expression levels of HBx, VEGF and CEACM1 and patient sex, age, liver cirrhosis, ATP, and significant correlation with tumor size, venous infiltration, intra-/extra-hepatic metastasis and capsule. In those HBV-related HCC patients with larger tumors, venous infiltration, metastasis, and no capsules, expression levels of HBx and VEGF were significantly elevated, while CEACAM1 expression was significantly decreased. A previous study indicated that CEACAM1 belonged to tumor suppressor factor. In TNM stage III to stage IV patients, the positive rate of CEACAM1 expression was significantly lower than those patients at stage I or II. Results suggested the inherent low-expression of CEACM1, thus may affect patient prognosis[19]. Tsukada et al[20] performed trial study on hepatoblastoma and found expression silencing in CEACAM1, with higher metastatic potency and unfavorable prognosis. Once with HBx expression, the adhesion ability of HCC cells was decreased significantly, with VEGF up-regulation, thus facilitating tumor progression under such micro-environment[21]. Tilki et al[22] performed a basic study using siRNA to induce CEACAM1 gene silencing in prostate cancer cell line and found significantly elevated VEGF expression. Meanwhile, the over-expression of CEACAM1 suppressed VEGF expression and decreased neo-angiogenesis in malignant tumors, as consistent with our data.

Conclusions

HBx, VEGF, and CEACAM1 were widely expressed in HBV-related HCC. HBx expression level was negatively correlated with CEACAM1 expression and was positively correlated with VEGF. For those HBV-related HCC patients with larger lesion, venous infiltration, metastasis, and no capsules, the expression levels of HBx and VEGF were significantly elevated while CEACAM1 was down-regulated. These results suggested that HBx might facilitate occurrence, invasion, migration, and progression of HBV-related HCC by inducing VEGF expression and inhibiting CEACAM1 expression with a series of signal pathways, although detailed mechanism requires further study for substantiation.

Conflict of interest

The authors declare no conflicts of interest.

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