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Clinical significance of herpes virus entry mediator expression in hepatitis B virus-related hepatocellular carcinoma

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Abstract. Herpes virus entry mediator (HVEM) is overexpressed in several malignancies, including hepatocellular carcinoma (HCC). However, to the best of our knowledge, the clinical significance of HVEM in hepatitis B virus (HBV)-related HCC remains unclear. Thus, the present study aimed to explore the clinical significance of HVEM in HBV-related HCC. In the present study, HVEM expression was evaluated in HCC cell lines and HCC frozen samples. The prognostic value of HVEM was assessed in a cohort of 221 patients with HBV-related HCC, following radical resection. B- and T-lymphocyte attenuator (BTLA) expression in subsets of $CD8^+$ T cells was determined via flow cytometry analysis. The results demonstrated high HVEM expression in HCC cell lines, and in HCC tissues compared with paired non-cancerous liver tissues. HVEM expression was demonstrated to be significantly associated with tumor encapsulation and vascular invasion. Furthermore, tumor HVEM status was significantly associated with infiltration of regulatory T cells, but not with $CD8^+$ T cells. Notably, high HVEM expression in HCC was determined to be an independent predictor of an unfavorable outcome of patients with HCC following radical resection. Higher BTLA expression (the receptor of HVEM) was observed in both HCC-infiltrating $CD8^+$ effector memory ($CCR7^-$ $CD45RA^-$) and $CD45RA^+$ effector memory ($CCR7^-$ $CD45RA^+$) T cells in HCC tissues and blood compared with those in paired peritumor tissues or peripheral blood. Taken together, the results of the present study suggest that HVEM may serve a critical role in HBV-related HCC, most likely by promoting tumor progression and tumor immune evasion, thus the HVEM/BLTA signaling pathway may be a potential target in tumor immunotherapy.

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Abbreviations: BTLA, B- and T-lymphocyte attenuator; CRC, colorectal cancer; DOI, density of interest; ESCC, esophageal squamous cell carcinoma; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HVEM, herpes virus entry mediator; OS, overall survival; PBMCs, peripheral blood mononuclear cells; PILs, peritumor-infiltrating lymphocytes; TILs, tumor-infiltrating lymphocytes; TNF, tumor necrosis factor; Tregs, regulatory T cells; TTR, time to recurrence

Key words: herpes virus entry mediator, B- and T-lymphocyte attenuator, hepatocellular carcinoma, prognosis, immunohistochemistry

Introduction

Liver cancer was the fourth leading cause of cancer-associated and the sixth most commonly diagnosed cancer worldwide in 2018 (1). Surgical resection, ablation and liver transplantation are the current curative treatment modalities for patients with HCC. However, a high rate of local recurrence or distant metastasis has impeded the improvement of patient outcome following curative treatments (2). Other treatments, such as transcatheter arterial chemoembolization and multi-tyrosine kinase inhibitor are used for advanced HCC (2). Recently, therapeutic monoclonal antibodies targeting immune checkpoint inhibitors (ICIs), such as the programmed cell death protein 1 (PD-1)/programmed death-ligand 1 (PD-L1) axis, have been approved in HCC treatment, and unprecedented improvement in tumor control has been reported (3). However, only a small subset of patients with HCC exhibit a marked response to a single antibody against ICIs, possibly due to the high complexity of the tumor microenvironment (4).

Herpes virus entry mediator (HVEM), a member of the tumor necrosis factor (TNF) receptor super family, interacts with B- and T-lymphocyte attenuator (BTLA), which activates its cytoplasmic domain that contains an inhibitory tyrosine-based motif, attenuating proliferation signals in antigen-activated lymphocytes (5). HVEM is widely expressed

on multiple cells, such as T, B, natural killer, dendritic and myeloid cells (6). Furthermore, the lung, liver and kidneys have been reported to express HVEM (7). In addition to BTLA, HVEM is also a ligand for CD160, and the TNF superfamily members LIGHT and lymphotoxin-β (6). HVEM interacts with CD160 or BTLA to mediate inhibitory signals in T cell proliferation and cytokine secretion (8). Conversely, ligation of HVEM with LIGHT mediates the activation of naïve T cells and clonal expansion (9). HVEM exhibits a bidirectional effect on T cell activation depending on the engaged ligands. However, the overall function of HVEM is inhibitory, based on the evidence that $HVEM^{-/-}$ T cells exhibit an enhanced activation profile and increased susceptibility to the development of Con A mitogen-induced, T cell-dependent autoimmune hepatitis and experimental autoimmune encephalopathy in HVEM-deficient mice (10). Ectopic HVEM expression has been demonstrated to be associated with obesity, autoimmune disease and inflammation (11,12). Furthermore, different types of tumor, including esophageal squamous cell carcinoma (ESCC), HCC and melanoma, exhibit higher HVEM expression in tumors compared with adjacent normal tissues (13-15). Downregulation of HVEM in ESCC cells induces cell cycle arrest and inhibits tumor growth *in vivo* (13). An inverse association between HVEM and tumor-infiltrating immune cells, including $CD4^+$, $CD8^+$ and $CD45RO^+$ lymphocytes has been reported in both human ESCC and hepatitis C virus (HCV)-related HCC (13,14). Notably, high HVEM expression in cancer serves as an independent predictor of poor survival outcomes in patients with ESCC or HCC, following radical resection (13,14). However, the expression status and clinical significance of HVEM in HCC with hepatitis B virus (HBV) remain largely unknown.

Thus, the present study aimed to investigate the clinical significance of HVEM in HBV-related HCC, and determine the association between HVEM and subsets of HCC-infiltrating immune cells. Furthermore, BTLA expression in subsets of $CD8^+$ T cells was investigated.

Materials and methods

Patients and specimens. The clinicopathological characteristics of patients with HCC included in the current study are presented in Table SI. For tissue microarray construction, 221 patients with a median age of 52 years and age range, 18-81 years, who underwent radical resection for HCC at the Department of Liver Surgery and Transplantation, Zhongshan Hospital (Shanghai, China) were enrolled between April 2002 and December 2007. The patients were comprised of 188 males and 33 females. The inclusion and exclusion criterion of the cohort are described in previous studies (16,17). The patient cohort was divided into the $HVEM^{high}$ group (≥50% of $HVEM^+$ tumor cells; n=139) and $HVEM^{low}$ group (<50% of $HVEM^+$ tumor cells; n=82). Follow-up was performed until mortality or May 2017. Patients were followed up every 2 months during the first postoperative year and then every 3 to 4 months for the remainder of the duration. The median follow-up for all patients was 53 months, with a range of 2-180 months. Time to recurrence (TTR) and overall survival (OS) time were calculated as the interval between primary surgical resection and the first recurrence or mortality, respectively. Briefly, the inclusion criteria were as follows: i) Underwent radical resection for HCC with distinctive pathological diagnosis; ii) no preoperative anticancer treatment or extrahepatic metastasis; and iii) complete follow-up data. Paired peripheral blood and fresh tissue samples were obtained from 20 patients with HCC randomly from the total patient cohort for lymphocyte isolation. Frozen HCC tissue samples and adjacent liver tissues (1 cm away from tumor tissues) from another 28 patients with HCC were randomly selected from the tissue bank of Zhongshan Hospital for PCR analysis. The present study was approved by the Zhongshan Hospital Research Ethics Committee (approval no. Y2017-186) and written informed consent was provided by all patients prior to the study.

Reverse transcription-quantitative (RT-q)PCR. Following radical resection of HCC tissue samples and adjacent liver tissues, RNA extraction was immediately performed, and both tissue samples and extracted RNA were preserved at -80˚C. Total RNA was extracted from frozen tissue samples using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse transcribed into complementary DNA at 45˚C (cDNA; 0.5 μg) using the cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). qPCR was subsequently performed using the TaqMan Universal PCR Master Mix (Thermo Fisher Scientific, Inc.) with 1 μl cDNA in a 25 μl final reaction volume and an ABI Prism 7300 system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following primer sequences were used for qPCR: HVEM forward, 5'-CTTGAGGCTGGTGCTGTATC-3' and reverse, 5'-GGTGGGCAATGTAGGTG-3'; and GAPDH forward, 5'-CACCCACTCCTCCACCTTTG-3' and reverse, 5'-CCA CCACCCTGTTGCTGTAG-3'. The following thermocycling conditions were used for qPCR: Initial denaturation at 95˚C for 10 min; 40 cycles of denaturation at 95˚C for 15 sec, annealing at 55˚C for 45 sec and extension at 60˚C for 15 sec. Relative HVEM expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (18) and normalized to the internal reference gene GAPDH.

Immunohistochemistry (IHC) and evaluation. Tissue microarray was constructed as previously described (16,17). The tissues were fixed by immersion in a 10% formalin solution for 4-8 h at room temperature. The thickness of sections was 5-15 μm. Briefly, following deparaffinization and rehydration in a graded series of ethanol (100, 95, 80 and 50%), sections were incubated with 0.3% H_2O_2 for 20 min at room temperature to inhibit endogenous peroxidase activity. Antigen retrieval was performed with Tris-ETDA (pH 9.0) using a microwave oven at 99-100˚C for 20 min. Subsequently, sections were blocked with 5% BSA (Sigma-Aldrich; Merck KGaA) at room temperature for 30 min, prior to incubation with primary antibodies directed against human HVEM/TNFRSF14 (ab47677; 1:20; Abcam), human CD8 (1:50; Dako; Agilent Technologies, Inc.) and human forkhead box P3 (FOXP3; cat. no. ab20034; 1:100; Abcam) overnight at 4˚C. A 100 μl of diluted biotinylated secondary antibody (1:1,000; cat. no. ab98624; Abcam) was then added and the sections were incubated in a humidified chamber at room temperature for 30 min. The components of the Histostain®-Plus kit (Invitrogen; Thermo Fisher Scientific, Inc.) were used for signal amplification and visualization.

Immunohistochemical staining for HVEM was blindly evaluated by two independent pathologists using confocal laser-scanning microscope (magnification, x100) (Olympus FluoView FV1000; Olympus Corporation), as previously described (13,15). Tumor cells (≥1,000) were counted and the percentage of tumor cells with positive staining was calculated. Integrated absorbance and the area in a photograph was measured using Image-Pro Plus software (version 6.0; Media Cybernetic, Inc.), in order to compare the expression levels of HVEM between HCC and peritumor tissue samples. The density of interest (DOI) was calculated as the product of integrated absorbance/total area. Tumor-infiltrating $CD8^+$ and $FOXP3^+$ T cells were counted in five randomly selected fields (magnification, x400) per sample by two independent investigators.

Cell lines. Human HCC cell lines, including HCCLM3, MHCC97H and MHCC97L [human HCC cell lines with high, moderate and low metastatic potential, respectively, which were derived from the same parental cell line and established in Liver Cancer Institute, Zhongshan Hospital, Fudan University (19,20)], and PLC (Japanese Cancer Research Bank) were used in the present study. All cell lines were maintained in DMEM (Thermo Fisher Scientific, Inc.) containing 10% FBS (Thermo Fisher Scientific, Inc.) supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin and incubated at 37˚C with 5% CO_2.

Immunocytochemistry. Immunocytochemistry was performed according to standardized protocols (21). Briefly, seed adheren T cells were cultured in 6-well tissue plates in a sterile tissue culture hood at room temperature overnight and then fixed with 4% formaldehyde for 10 min at room temperature and permeabilized using 0.2% Triton X-100 at room temperature for 15 min. Subsequently, cells were blocked with 1% BSA for 30 min at room temperature, prior to incubation with anti-human HVEM/TNFRSF14 antibody (1:10; cat. no. ab47677; Abcam) overnight at 4˚C. Next, the cells were incubated with the secondary antibody (1:2,000; cat. no. ab205718; Abcam) for 1 h at room temperature. The components of the Alexa Fluor Plus 488 (Invitrogen; Thermo Fisher Scientific, Inc.) were used for signal amplification, and DAPI at 300 ng/ml incubated for 10 min away from light was used as a nuclear counterstain. The slides were observed under a confocal laser-scanning microscope (magnification, x100) (Olympus FluoView FV1000; Olympus Corporation).

Cell isolation and flow cytometric analysis. Isolation of peripheral blood mononuclear cells (PBMCs), peritumor-infiltrating lymphocytes (PILs) and tumor-infiltrating lymphocytes (TILs) was performed as previously described (16). Briefly, PBMCs were isolated by Ficoll density gradient centrifugation, according to the manufacturer's protocol (GE Healthcare). PILs and TILs were isolated from clinical HCC specimens by Percoll gradient centrifugation as previously described (22). Analysis of surface antigen expression was performed, according to the manufacturer's protocol (BD Biosciences). Briefly, BTLA expression on $CD8^+$ T cells was investigated in relation to the differentiation stage discriminated by the expression of chemokine receptor CCR7 in combination with the naïve cell marker CD45RA. $CD8^+$ T cells were gated as follows: i) Naïve ($CCR7^+$ $CD45RA^+$); ii) central memory (TCM, $CCR7^+$ $CD45RA^-$); iii) effector memory ($CCR7^-$ $CD45RA^-$); and iv) effector memory RA ($CCR7^-$ $CD45RA^+$). $1x10^6$ PBMCs, PILs and TILs were washed in PBS supplemented with 1% BSA and 0.1% sodium azide (Sigma-Aldrich; Merck KGaA) three times, and incubated with anti-BTLA (1:10; cat. no. 746759, BD Pharmingen) for 30 min at 4˚C. Following washing three times using ice cold PBS, 10% FCS, 1% sodium azide and centrifugation at 400 x g at 4˚C for 5 min, samples were fixed in PBS/1% paraformaldehyde (Sigma-Aldrich; Merck KGaA) overnight at 4˚C. Lymphocytes were gated based on forward and side scatters, and at least $1x10^5$ gated events were acquired for each sample and analyzed using FlowJo v.10.5.3 software (FlowJo LLC).

Statistical analysis. Statistical analysis was performed using SPSS software (version 16.0; SPSS, Inc.). Paired Student's t-tests were used for comparing two groups. A χ^2 test was used to determine the association between HVEM expression and clinicopathological characteristics of patients with HCC. One-way ANOVA followed by Bonferroni's post-hoc test was used to compare differences between multiple groups. Univariate survival analysis was performed using the Kaplan-Meier method and the log-rank test, while Cox multivariate analysis was performed to adjust for potential confounding variables in a stepwise manner (forward, likelihood ratio) and determine the independent prognostic factors. Data are shown as the mean ± standard deviation of three independent experiments, and t-tests were used to compare group averages. $P<0.05$ was considered to indicate a statistically significant difference.

Results

HVEM expression in HBV-related HCC and HCC cell lines. RT-qPCR was performed to determine HVEM expression in patients with HCC with a background of HBV. The results demonstrated that HVEM expression was significantly higher in HCC tissues compared with paired peritumor tissues (P=0.0066; Fig. 1A). Furthermore, HVEM expression was assessed in HCC via IHC analysis. Positive staining of HVEM was identified predominantly on the membrane and in the cytoplasm of tumor cells. Scatter positive staining of the stromal cells was also observed in peritumor tissue, and accidentally observed embolus also demonstrated higher HVEM expression compared with surrounding liver tissue (Fig. 1B). HCC tissue was demonstrated to have a significantly stronger HVEM expression intensity when evaluated by DOI (0.035±0.021 vs. 0008±0.009; P<0.0001; Fig. 1C). Furthermore, high HVEM expression was demonstrated in HCC cell lines via immunocytochemistry analysis (Fig. 1D).

Association between HVEM expression and clinicopathological characteristics of HCC. The patient cohort was divided into the $HVEM^{high}$ group (≥50% of $HVEM^+$ tumor cells, n=139) and $HVEM^{low}$ group (<50% of $HVEM^+$ tumor cells, n=82), as previously described (10,11). Vascular invasion was significantly higher and tumor encapsulation was significantly lower in the $HVEM^{high}$ group compared with

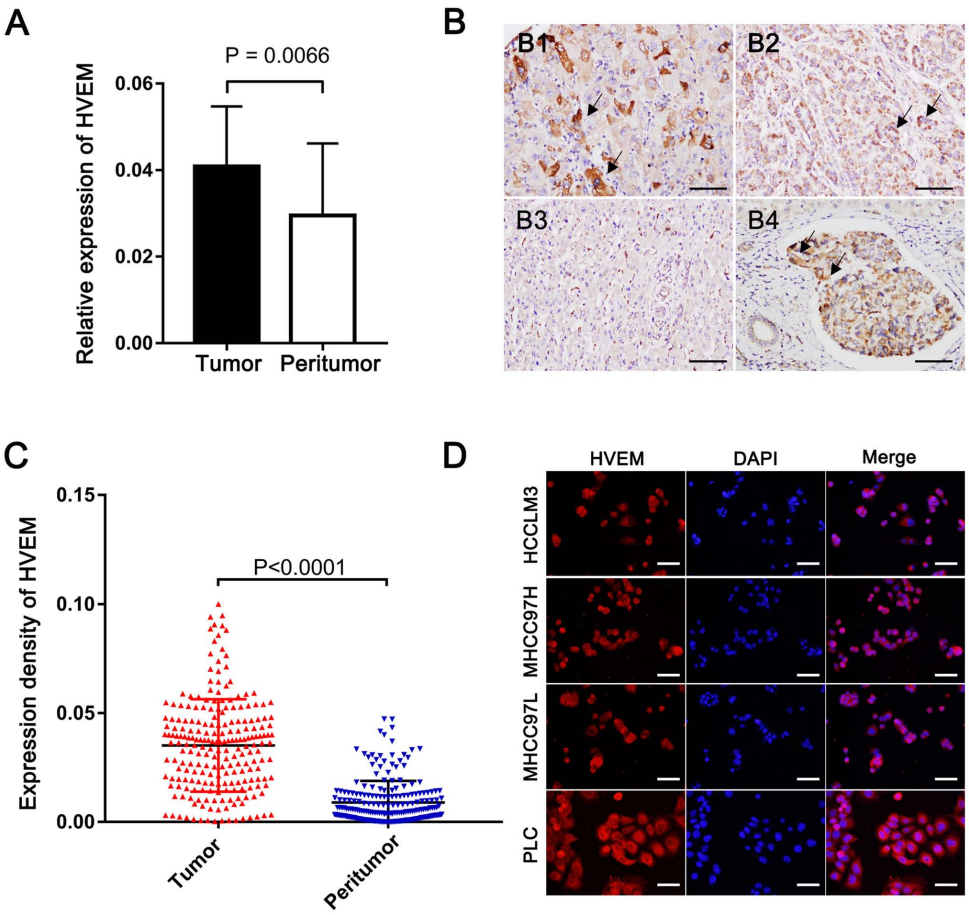

Figure 1. HVEM expression in surgically resected HCC tissues. (A) Reverse transcription-quantitative PCR analysis demonstrated that HVEM expression was significantly higher in HCC tissues compared with peritumoral liver tissues (n=20). (B) Representative images of HVEM expression in HCC. (B-1 and B-2) Positive staining of HVEM was predominantly exhibited on the cytomembrane and in the cytoplasm of tumor cells and is indicated by the black arrows. (B-3) Peritumor tissues demonstrated relatively low HVEM expression. (B-4) High HVEM expression in tumor embolus was observed and is indicated by the black arrows. Scale bar, 50 μm. (C) Density of HVEM staining was evaluated by density of interest (mean ± standard deviation). (D) Immunocytochemical staining of HVEM in HCC cell lines. DAPI was used as a nuclear counterstain. Scale bar, 50 μm. HVEM, herpes virus entry mediator; HCC, hepatocellular carcinoma.

the HVEM^{low} group (P=0.048 and P=0.036, respectively; Table I). Furthermore, tumors with high HVEM expression had a relatively higher rate of advanced BCLC stage; however, no significant difference was observed between the groups (P=0.087; Table I). AFP level and TNM stage failed to demonstrate a prognostic value for patients with HCC (P=0.081 and P=0.211, respectively; Table I). Taken together, these results suggest that HVEM may be involved in disease progression of patients with HCC.

Association between HVEM and FOXP3$^+$ T cells in patients with HCC. HVEM may be involved in tumor progression by inducing the immune escape of tumor cells (23). The present study evaluated the associations between HVEM with CD8$^+$ T cells and FOXP3$^+$ regulatory T cells (Tregs). High FOXP3 expression was significantly higher in the HVEM^{high} group compared with the HVEM^{low} group (P=0.005); however, no significant association was observed between HVEM expression and CD8$^+$ T cells (Table I).

Prognostic significance of HVEM in HBV-related HCC. Kaplan-Meier analysis demonstrated that high HVEM expression in HCC tissue was associated with a shorter TTR time and shorter OS time compared with the HVEM^{low} group (P<0.001; Fig. 2). Factors that demonstrated significance by univariate analysis presented in Table II were enrolled in a multivariate Cox proportional hazards model. We found that α-fetoprotein, γ-glutamyl transferase, tumor size, vascular invasion, FOXP3 and CD8 are independent prognostic factor for predicting OS. Importantly, HVEM was revealed to be an independent prognostic factor for predicting recurrence and OS (Table II).

BTLA expression in CD8$^+$ T cells. CD8$^+$ T cells act as the main effector cells in the tumor microenvironment (24). As expected, the infiltration of CD8$^+$ T cells decreased in HCC compared with that in paired peritumor tissue and peripheral blood (Fig. 3A). The ligation of BTLA by HVEM expressed by HCC cells may result in decreased T cell proliferation and cytokine secretion (25). Thus, BTLA expression on CD8$^+$ T cells was investigated in relation to the differentiation stage discriminated by the expression of chemokine receptor CCR7 in combination with the naïve cell marker CD45RA. CD8$^+$ T cells were gated as follows: i) Naïve (CCR7$^+$ CD45RA$^+$); ii) central memory (TCM, CCR7$^+$ CD45RA$^-$); iii) effector memory (CCR7$^-$ CD45RA$^-$) and iv) effector memory RA

Table I. Association between HVEM expression and clinicopathological characteristics of patients with hepatocellular carcinoma (n=221).

Characteristic	Number of patients, n	HVEM expression		P-value
		Low, n	High, n	
Age, years				>0.999
≤52	110	69	41	
>52	111	70	41	
Sex				0.050
Male	188	113	75	
Female	33	26	7	
HBV history				0.606
Yes	204	127	77	
No	17	12	5	
Liver cirrhosis				0.534
Yes	193	123	70	
No	28	16	12	
AFP, ng/ml				0.081
≤20	79	56	23	
>20	142	83	59	
γ-GT, U/l				0.576
≤56	98	64	34	
>56	123	75	48	
Tumor size, cm				0.051
≤5	114	79	35	
>5	107	60	47	
Tumor number				>0.999
Single	172	108	64	
Multiple	49	31	18	
Tumor encapsulation				0.036
None	113	79	34	
Complete	108	60	48	
Tumor differentiation				0.205
I-II	131	87	44	
III-IV	90	52	38	
Vascular invasion				0.048[a]
Yes	130	89	41	
No	91	50	41	
TNM stage				0.211
I	107	72	35	
II-III	114	67	47	
BCLC stage				0.087
A	80	57	23	
B	50	32	18	
C	91	50	41	
FOXP3				0.005
Low	117	84	33	
High	104	55	49	
CD8				0.676
Low	108	66	42	
High	113	73	40	

HVEM, herpes virus entry mediator; HBV, hepatitis B virus; AFP, α-fetoprotein; γ-GT, γ-glutamyl transferase; TNM, tumor-node-metastasis; BCLC, Barcelona clinic liver cancer; FOXP3, forkhead box protein 3. TNM stage, BCLC stage and tumor differentiation were based on previous studies (39-41).

Table II. Univariate and multivariate analyses of prognostic factors in patients with hepatocellular carcinoma (n=221).

	Overall survival time			Time to recurrence		
	Univariate	Multivariate analysis		Univariate	Multivariate analysis	
Factor	P-value	HR (95% CI)	P-value	P-value	HR (95% CI)	P-value
α-fetoprotein, ng/ml (≤20 vs. >20)	0.001	1.553 (1.054-2.288)	0.026	0.066	NA	NA
γ-glutamyl transferase, units/l (≤56 vs. >56)	<0.001	1.845 (1.297-2.625)	0.001	0.089	NA	NA
Liver cirrhosis (No vs. Yes)	0.033	NS	NS	0.076	NA	NA
Tumor size, cm (≤5 vs. >5)	<0.001	1.436 (1.006-2.050)	0.046	0.002	NS	NS
Tumor multiplicity (Single vs. Multiple)	0.001	NS	NS	0.001	1.785 (1.217-2.617)	0.003
Tumor encapsulation (Complete vs. None)	0.009	NS	NS	0.003	NS	NS
Tumor differentiation (I/II vs. III/IV)	0.082	NA	NA	0.248	NA	NA
Vascular invasion(No vs. Yes)	<0.001	2.974 (2.057-4.298)	<0.001	<0.001	2.895 (2.037-4.117)	<0.001
HVEM (Positive vs. Negative)	<0.001	2.162 (1.528-3.059)	<0.001	0.003	1.752 (1.222-2.513)	0.002
FOXP3 (High vs. Low)	<0.001	2.314 (1.630-3.284)	<0.001	0.013	NS	NS
CD8 (High vs. Low)	0.001	0.523 (0.367-0.746)	<0.001	0.084	NA	NA

The Kaplan-Meier method and log-rank test was used for univariate analysis, while the Cox multivariate proportional hazards regression model, with a stepwise manner (forward, likelihood ratio) was adopted for subsequent analysis. HR, hazard ratio; CI, confidence interval; HVEM, herpes virus entry mediator; FOXP3, forkhead box protein 3; NS, not significant; NA, not applicable.

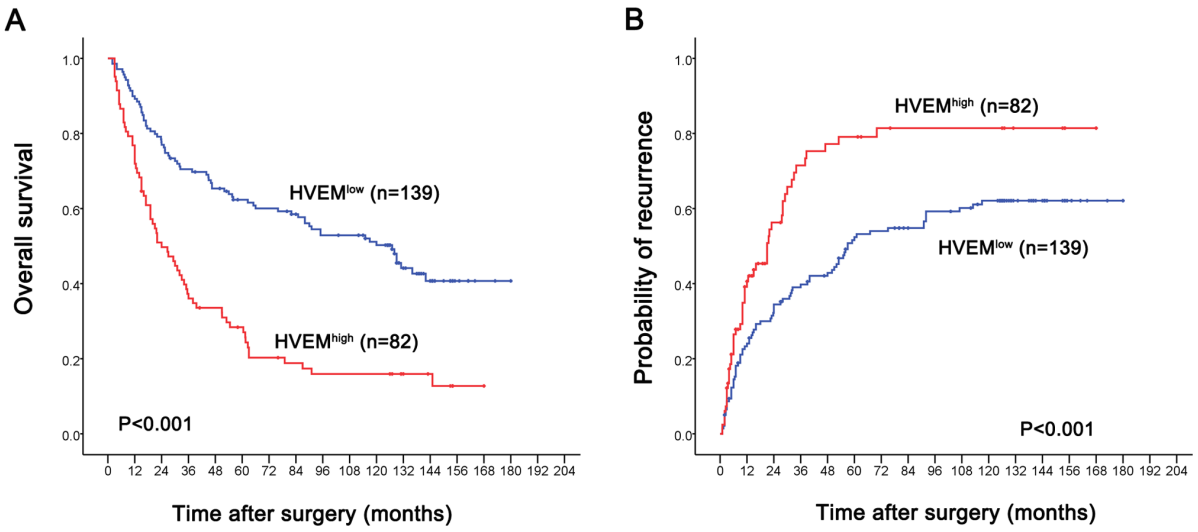

Figure 2. Clinical significance of HVEM in patients with hepatocellular carcinoma following radial resection. (A) The HVEM^{high} group had a shorter overall survival time compared with the HVEM^{low} group. (B) The probability of recurrence was higher in the HVEM^{high} group compared with the HVEM^{low} group. P<0.001. HVEM, herpes virus entry mediator.

(CCR7$^-$ CD45RA$^+$). The results demonstrated that the surface expression of BTLA in these cell subtypes gradually decreased with differentiation stage (Fig. 3A). However, HCC-infiltrating CD8$^+$ T cells still exhibited persistently high levels of BTLA. Specifically, both effector memory and effector memory RA CD8$^+$ T cells exhibited higher levels of BTLA in HCC tissues compared with peritumor tissues (Fig. 3B).

Discussion

It has been demonstrated that HVEM plays dynamic immune regulatory functions in various physiological and pathological conditions (6,12). In the tumor microenvironment, HVEM is involved in tumor immune evasion through ligation with BTLA, a coinhibitory receptor with functional similarities to PD-1 and CTLA-4 (3). The present study demonstrated a significantly higher expression of HVEM in HCC compared with paired peritumor tissue. Furthermore, high HVEM expression was associated with poor clinical outcome and invasive characteristics, such as high rate of vascular invasion and infiltration of suppressive Tregs. It has been reported that HCC-infiltrating CD8$^+$ T cells differentiate from naïve to effector cells, and these cells were important in HCC and were demonstrated to persistently express high levels of BTLA (26),

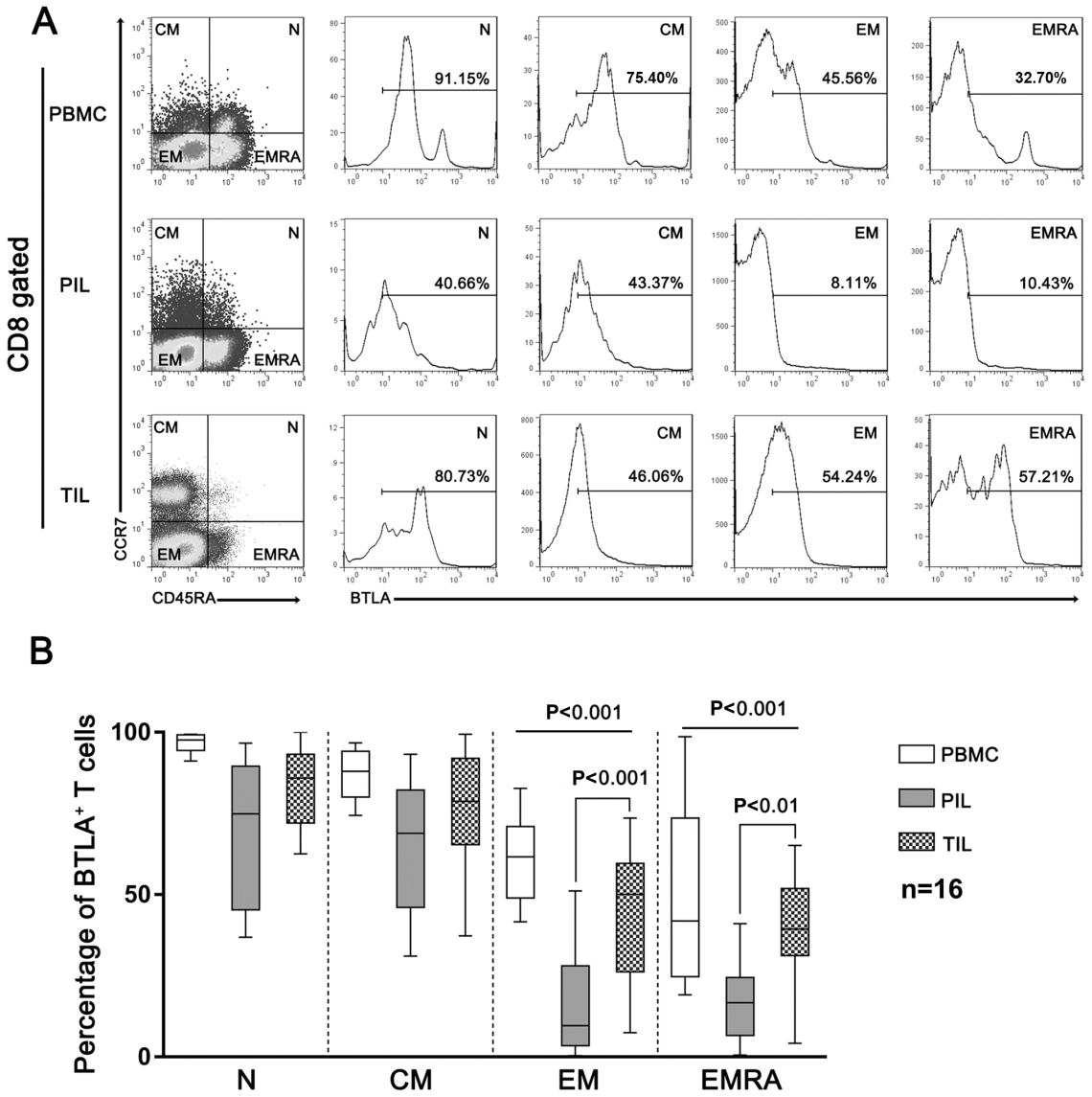

Figure 3. BTLA expression in HCC-infiltrating CD8$^+$ T cells. (A) Representative figures of BTLA expression in CD8$^+$ T cells from peripheral blood, peritumor tissue and tumor tissue of patients with HCC. CD8$^+$ T cells were divided into four subsets according to CD45RA and CCR7 expression (N, CCR7$^+$ CD45RA$^+$; CM, CCR7$^+$ CD45RA$^-$; EM, CCR7$^-$ CD45RA$^-$ and; EMRA, CCR7$^-$ CD45RA$^+$). BTLA$^+$ T cells were gated from BTLA$^-$ T cells using an established threshold, according to the autologous naïve T cell subsets of PBMC, which is always BTLA positive. (B) Statistical analysis of BTLA$^+$CD8$^+$ T cell subsets in PBMCs, PILs and TILs derived from patients with HCC. HCC, hepatocellular carcinoma; CD8, cluster of differentiation 8; BTLA, B- and T-lymphocyte attenuator; PBMCs, peripheral blood mononuclear cells; PILs, peritumor-infiltrating lymphocytes; TILs, tumor-infiltrating lymphocytes; N, naïve; CM, central memory, EM, effector memory; EMRA, effector memory RA$^+$.

which underlines the importance of the HVEM/BTLA signaling pathway in HCC.

Immunotherapy based on ICIs has reported promising therapeutic outcomes in patients with cancer (27). PD-1/PD-L1 and CTLA-4 inhibitors have been approved for certain cancer treatments, and some are currently under clinical trials (4). However, the low response rate is one of the major difficulties for ICI-based treatments in some patients with cancer (28). In HCC, it has been reported that only 10-30% of treated patients respond to anti-PD-1/PD-L1 therapy (29). Conversely, several other ICIs, including HVEM/BTLA, CD73 and mucin domain 3, also regulate immune responses in tumor niches and may be alternative targets for novel immune therapy (3). The HVEM/BTLA signaling pathway is considered a novel target for checkpoint blockade, based on the fact that HVEM/BTLA inhibition enhances human T cell responses when used alone or in combination with anti-PD-1 treatment (30-33). It is reasonable to expect the combination of HVEM blockade and other anticancer treatments, such as resection, ablation, chemotherapy and anti-PD-1/PD-L1 treatment, may induce a synergistic anticancer effect. However, clinical trials are required to effectively evaluate the combined modality.

HVEM is involved in cancer progression through mediating immune evasion; a higher expression of HVEM in cancer tissue is associated with relatively poorer survival outcomes, as reported in patients with ESCC,

HCV-related hepatocellular carcinoma and colorectal cancer (CRC) (13,14,34). However, to the best of our knowledge, the significance of HVEM in HBV-related HCC was previously undetermined. The present study verified the prognostic role of HVEM in HBV-related HCC. Furthermore, a significant association between HVEM and aggressive biological behavior of HCC, including vascular invasion and incomplete tumor capsule, was identified. Similarly, overexpression of HVEM in patients with non-small cell lung carcinoma of N2 lymph node metastasis or late-stage has been observed (24). In CRC and gastric cancer, HVEM status is significantly associated with tumor status and pathological stage (34,35). Conversely, the present study determined that HVEM expression levels are associated with tumor-infiltrating Tregs, a robust immune inhibitor, rather than $CD8^+$ T cells (36). Notably, Tao *et al* (36) reported that Tregs exert their suppressive effect via the upregulation of HVEM, which, upon ligation with BTLA expressed on effector cells, helps control immune response. $HVEM^{-/-}$ Tregs have been demonstrated to decrease suppressive activity compared with wild-type Tregs (36). These findings suggest that immunotherapy targeting HVEM may lead to activation of effector T cells and dampening of Tregs.

Studies have reported that HVEM can activate BTLA, thus inhibiting $CD8^+$ T cell differentiation and cytokine secretion (37,38). The present study identified aberrant persistent high expression of BTLA by differentiated effector T cells derived from HCC tissue, suggesting the HVEM/BTLA signaling pathway may play a role in the inhibition of efficient immune responses against cancer.

Overall, the results of the present study suggest a prognostic value of HVEM in patients with HCC following radical resection. Thus, the HVEM/BTLA signaling pathway may be a target in cancer immunotherapy.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

YY and SJQ designed the present study and contributed to the development of methodology. YY and JLH contributed to the acquisition of data. XCN and GL performed the experiments. JLH, WG, PYZ, RYG, CZ, YRY and BYS analyzed the data and performed the studies. YY drafted the manuscript and reviewed the manuscript for important intellectual content and SJQ acquired the funding. YY contributed to the administrative, technical, or material support. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Zhongshan Hospital Research Ethics Committee (approval no. Y2017-186), and written informed consent was provided by all patients prior to the study start.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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