

HVEM Expression Contributes to Tumor Progression and Prognosis in Human Colorectal Cancer

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Abstract. *Background: Herpesvirus entry mediator (HVEM) has been recently suggested to play certain roles in cancer biology. We examined HVEM expression in human colorectal cancer (CRC) to reveal its clinical importance. Materials and Methods: Immunohistochemical staining was carried-out in normal epithelium, benign and malignant lesions. Results: While intense HVEM expression was not observed in normal epithelium and hyperplastic polyps, 24% of adenoma and more than half of CRCs had high HVEM expression. In 234 CRCs, HVEM expression was significantly associated with tumor status and pathological stage. Patients with high HVEM expression had a significantly poorer prognosis than those with low expression. Importantly, HVEM status had an independent prognostic value in CRC. Furthermore, HVEM status was inversely corrected with the presence of tumor-infiltrating T-cells. Conclusion: HVEM may play a critical role in tumor progression and immune evasion, and may also be a novel prognostic marker and potential therapeutic target in human CRC.*

Colorectal cancer (CRC) is the third cause of cancer-related death worldwide (1). In the past decade, there has been significant progress in the treatment of CRC, including chemotherapy and molecular-targeted therapy. Despite recent advances in treatment, the prognosis of advanced or recurrent CRC remains poor (2-4). Thus, novel approaches still need to be developed and established to improve the prognosis of patients with CRC.

Several members belonging to the tumor necrosis factor receptor (TNFR) and immunoglobulin (Ig) superfamily

regulate lymphocyte activation (5, 6). Herpesvirus entry mediator (HVEM; TNFRSF14) is a member of the TNFR superfamily which is expressed on a wide range of hematopoietic cells including T-cells, B-cells, natural killer cells, monocytes, and immature dendritic cells. HVEM is also expressed in various nonhematopoietic cells and tissues including the lung, liver, and kidney (7-9). HVEM ligands belong to two distinct families: the TNF-related cytokines, lymphotoxin-related inducible ligand that competes for glycoprotein D binding to herpesvirus entry mediator on T cells (LIGHT) and lymphotoxin- α , and the Ig-related membrane proteins, B and T lymphocyte attenuator (BTLA) and cluster of differentiation 160 (CD160) (9-11). The binding of HVEM to LIGHT stimulates the host immune response. In sharp contrast, HVEM can provide an inhibitory signal to T-cells upon binding to BTLA or CD160 (6, 9). Despite the complexity of HVEM signaling, its inhibitory function seems to be dominant as demonstrated in HVEM-deficient mice (12). Previous studies have focused on the HVEM pathway in inflammatory and immunological disease conditions such as autoimmune disease, inflammatory bowel disease and transplantation (12-16). We and others have also reported HVEM expression in various tumor cell lines and clinical tumor tissues, including breast and esophageal cancer, chronic lymphocytic leukemia and melanoma (9, 17-19). These studies have demonstrated that HVEM might be involved in tumor progression and evasion of immune response. However, to our knowledge, the clinical significance of HVEM in human CRC tissues has not been previously addressed. Therefore, the aim of the present study was to investigate the involvement of HVEM in tumor biology and immunity in human CRC.

Materials and Methods

Patients. We examined 234 patients with CRC who underwent surgery at the Department of Surgery, Nara Medical University, Japan between 2004 and 2007. The median age of the patients was 69.5 years, with a range of 35 to 94 years. Tissues were obtained from the resected specimens, and each specimen was fixed in 10% phosphate-buffered formalin and embedded in paraffin. A serial

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section from each specimen was stained with hematoxylin and eosin stain for histological evaluation. Tumors were classified according to the tumor-node-metastasis staging system (20). The median follow-up for all patients was 60.5 months, with a range of 0.5 to 87 months. Furthermore, we examined 10 patients with hyperplastic polyp and 50 patients with adenoma who underwent endoscopic resection at our institution. Written informed consent was obtained from all patients according to our institutional guidelines.

Immunohistochemistry. Immunohistochemical staining was carried out using the Dako Envision system (Dako Cytomation, Kyoto, Japan) as previously described (21). As primary antibody, antibodies to HVEM (94804; R&D System, Minneapolis), CD4 (4B12; Dako Cytomation), and CD8 (C8/144B; Dako Cytomation) were used. Paraffin-embedded tissues were de-paraffinized and rehydrated in a graded series of ethanols. Antigen retrieval was performed by heating tissue sections using a target retrieval solution, pH 9.0 (Dako). After neutralization of endogenous peroxidase, the sections were incubated with each primary antibodies overnight at 4°C. After three washes in phosphate-buffered saline (PBS), the sections were incubated with secondary antibodies at 37°C for 0.5 (HVEM and CD8) or 1 (CD4) h. After three washes in PBS, the sections were visualized with diaminobenzidine (DAB) (Dako) for approximately 5-10 min. The slides were counterstained with hematoxylin, dehydrated in ethanol.

Evaluation of immunostaining. Immunohistochemistry for HVEM was evaluated according to its intensity and the percentage of positively-stained tumor cells in a blinded manner. Five fields were randomly selected. The percentage of positively-stained tumor cells in each selected field was determined by counting individual tumor cells at high magnification. At least 200 tumor cells were scored per $\times 200$ field, and the median number for each sample was calculated. The intensity was classified into four groups: none: 0 point; weak: 1 point; intermediate: 2 points and strong: 3 points (Figure 1). The percentage of positively-stained tumor cells was classified into four groups as follows: 0-25%: score 1; 26-50%: score 2; 51-75%: score 3; and 76-100%: score 4. We then evaluated HVEM expression in each tissue according to the total score by adding the points and scores for each (total number; 1-7). Specimens with a total score of 1-5 were classified as having low HVEM expression, and those with 6-7 were classified as having high HVEM expression. Immunohistochemistry for CD4⁺ and CD8⁺ T-cells was evaluated by counting the number of tumor-infiltrating T-lymphocytes (TILs) as previously described (22).

Statistical analysis. The significance of the difference in HVEM expression by several clinicopathological variables was assessed by the Student's *t*-test, the Chi-square test or Fisher's exact test as appropriate. The overall survival time was calculated from the date of surgery to the date of death. The Kaplan-Meier method was used to estimate the probability of survival, and significance was assessed by the log-rank test. Univariate and multivariate analyses were performed by the Cox proportional hazards model to evaluate significant prognostic predictors and their relative role. We use the terms tumor status as T factor, nodal status as N factor, and metastatic status as M factor in the tumor-node-metastasis classification, retrospectively. A *p*-value <0.05 was considered statistically significant.

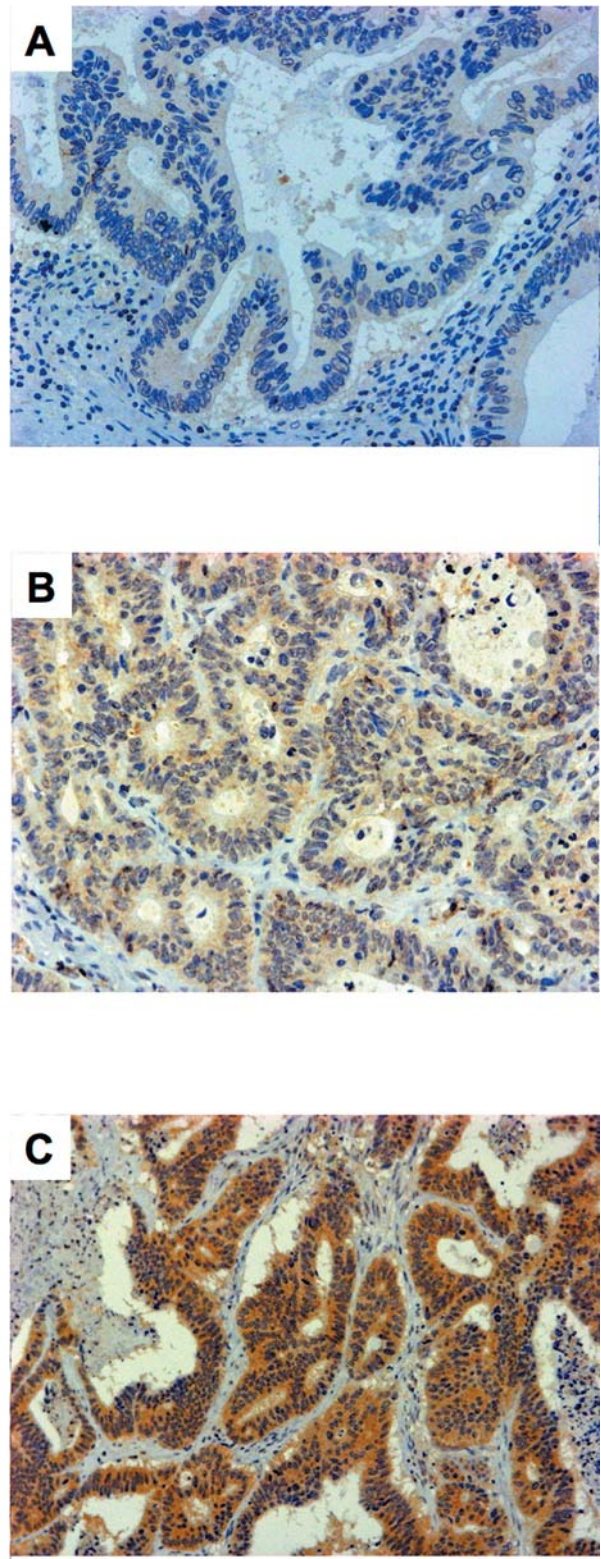


Figure 1. Immunohistochemical staining of herpesvirus entry mediator (HVEM) in human colorectal cancer tissues. Representative case of tumors with weak (A), intermediate (B) and strong (C) HVEM expression. Original magnification, $\times 200$.

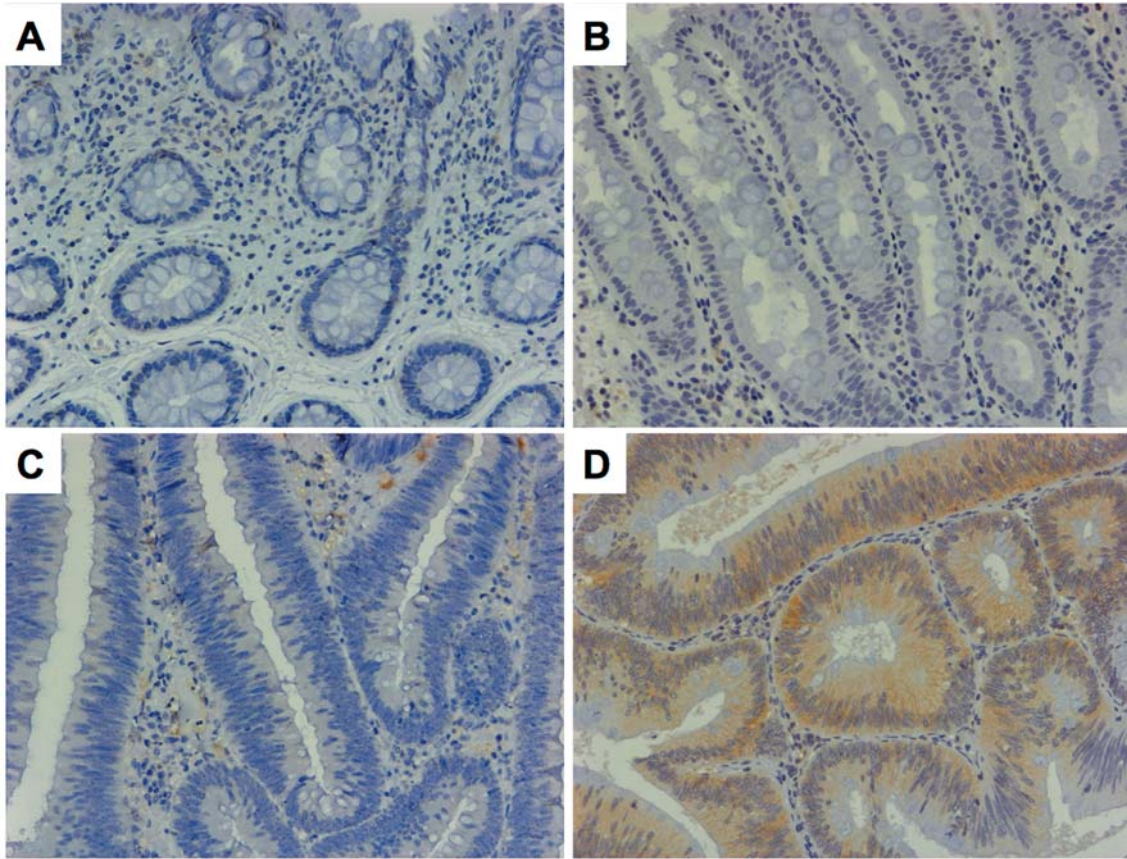


Figure 2. Immunohistochemical staining of herpesvirus entry mediator (HVEM) in normal colon epithelium, hyperplastic polyp and adenoma. Representative case of normal colonic epithelium with low HVEM (A), hyperplastic polyp with low HVEM (B), adenoma with low HVEM (C) and adenoma with high HVEM (D). Original magnification, $\times 200$.

Results

HVEM expression in human normal and neoplastic colon tissues. To investigate the involvement of HVEM in CRC, we first examined HVEM expression in various colonic tissues including normal epithelium, and benign and malignant lesions by immunohistochemical analysis.

Among 10 samples of normal colonic epithelium examined, 3 had no staining for HVEM expression and 7 had a weak staining with score 1 at the protein level (Figure 2A). Out of the 10 hyperplastic polyps examined for HVEM expression, 5 had no staining, three weak, and 2 had an intermediate staining with score 1 (Figure 2B). Therefore, all normal epithelium and hyperplastic polyps were classified as having low HVEM expression (Table I). In 50 adenomas, 16% (8/50) showed no staining, 28% (14/50) weak, 42% (21/50) intermediate and 14% (7/50) strong staining for HVEM (Figure 2C and D). Furthermore, 54% (27/50) were defined as having score 1, 10% (5/50) with score 2, 8% (4/50) with score 3 and 28% (14/50) with score 4. As a

result, 76% were classified as having low and 24% as having high HVEM expression (Table I).

In the present study, a total of 234 patients with CRC were evaluated for tumor HVEM expression. HVEM expression was identified in both the cytoplasm and plasma membrane of cancer cells (Figure 1). Among 234 CRCs, 5.1% had no staining, 26.1% weak, 49.6% intermediate and 19.2% strong staining for HVEM. In addition, 13.7% were defined with score 1, 16.2% with score 2, 20.0% with score 3 and 50.0% with score 4. As a result, 49.1% of patients with CRC were classified as having low HVEM expression and 50.9% as having high HVEM expression (Table I).

Correlation of tumor HVEM expression with clinicopathological factors. We then examined the correlation between tumor HVEM expression and various clinicopathological factors (Table II). As a result, tumors with high HVEM expression had more advanced tumor status and pathological stage. In contrast, there were no significant correlations of HVEM expression with other factors, including

Table I. *Herpesvirus entry mediator (HVEM) expression in human colonic tissues.*

	No.	HVEM expression	
		Low, n (%)	High, n (%)
Normal epithelium	10	10 (100)	0 (0)
Hyperplastic polyp	10	10 (100)	0 (0)
Adenoma	50	38 (76.0)	12 (24.0)
Colorectal cancer	234	115 (49.1)	119 (50.9)

Table II. *Relationship between clinicopathological characteristics and herpesvirus entry mediator (HVEM) status in colorectal cancer.*

Characteristics	HVEM expression			p-Value
	Total	Low	High	
	n=234	n=115 (%)	n=119 (%)	
Age at surgery (years)				
≤70	117	55 (47.8)	62 (52.1)	0.513
>70	117	60 (52.2)	57 (47.9)	
Gender				
Male	151	78 (67.8)	73 (61.3)	0.300
Female	73	37 (32.2)	46 (38.7)	
Tumor location				
Colon	140	67 (58.2)	73 (61.4)	0.889
Rectosigmoid	27	14 (12.2)	13 (10.9)	
Rectum	67	34 (29.6)	33 (27.7)	
Histopathological grading				
G1	105	56 (48.7)	49 (41.2)	0.635
G2	120	54 (47.0)	66 (55.5)	
G3	9	5 (4.3)	4 (3.3)	
Tumor status				
T1, T2	60	38 (33.0)	22 (18.5)	0.011
T3, T4	174	77 (67.0)	97 (81.5)	
Nodal status				
N0	110	58 (50.4)	52 (43.7)	0.585
N1	80	37 (32.2)	43 (36.1)	
N2	44	20 (17.4)	24 (20.2)	
Metastatic status				
M0	207	103 (89.6)	104 (87.4)	0.603
M1	27	12 (10.4)	15 (12.6)	
Pathological stage				
I	45	32 (27.8)	13 (10.9)	0.001
II, III, IV	189	83 (72.2)	106 (89.1)	

nodal status, metastatic status, age, gender, tumor location, histopathological grade.

Prognostic value of HVEM expression in human CRC. Among the 234 patients with CRC, the patients with high HVEM expression had significantly poorer survival than those with low HVEM expression (Figure 3). Furthermore, the univariate analysis showed that HVEM status, as well as age,

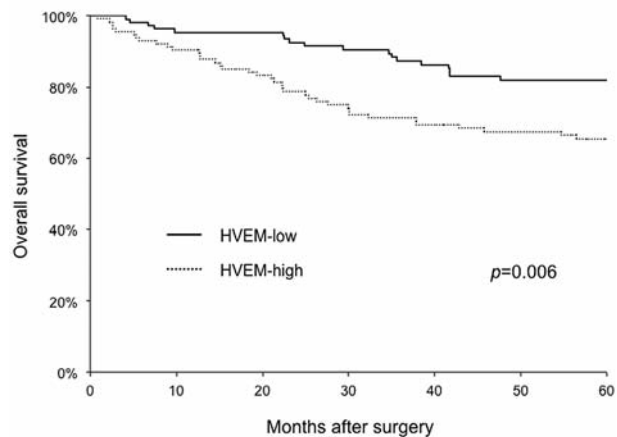


Figure 3. *Overall survival of 234 patients with colorectal cancer in relation to their tumor herpesvirus entry mediator (HVEM) status. Patients with high HVEM expression had a significantly poorer prognosis than those with low expression.*

histopathological grading, tumor status, nodal status, and metastatic status, were significant prognostic factors (Table III). In addition, the multivariate analysis indicated that HVEM status, as well as age, nodal status and metastatic status, were significant independent prognostic factors (Table III).

Inverse correlation between tumor HVEM expression and the presence of TILs. Finally, we investigated the correlation between tumor HVEM expression and the presence of TILs by immunohistochemical analysis. As a result, we found a significant inverse correlation between tumor HVEM expression and TILs, including both CD4⁺ and CD8⁺ T-cells (Figure 4). These data suggested that tumor HVEM expression might be involved in tumor immune evasion in human CRC.

Discussion

Tumors evade host immune response through several mechanisms. One of the critical mechanisms is the inhibition of antitumor T-cell activation by expression of immunosuppressive proteins on the tumor surface (23, 24). The B7/cytotoxic T-lymphocyte antigen-4 (CTLA4) pathway and the programmed cell death 1 ligand 1 (PD-L1)/programmed cell death protein 1 (PD1) pathway are known to be major negative regulatory T-cell pathways (25, 26). Recent studies have shown that other molecules and pathways might also play critical roles in tumor immunity (5, 27). Among them, HVEM has been suggested to play a potentially important role in several human malignancies (9, 17-19). However, there is scarce knowledge about the clinical importance of HVEM in actual human cancer.

Table III. Univariate and multivariate prognostic analysis of 234 patients with colorectal cancer.

Characteristic	Univariate analysis			Multivariate analysis		
	HR	95% CI	<i>p</i> -Value	HR	95% CI	<i>p</i> -Value
Age (≥ 70 vs. < 70 years)	1.869	1.121-3.116	0.017	1.777	1.054-2.995	0.031
Sex (male vs. female)	1.094	0.653-1.832	0.734			
Tumor location (RaRb vs. C-RS)	0.983	0.575-1.681	0.951			
Histopathological grading (G2-G3 vs. G1)	2.350	1.362-4.054	0.002	1.432	0.817-2.512	0.210
Tumor status (T3-T4 vs. T1-T2)	4.742	1.900-11.83	0.001	2.312	0.889-6.014	0.086
Nodal status (N1-N2 vs. N0)	2.933	1.665-5.166	< 0.001	2.015	1.122-3.622	0.019
Metastatic status (M1 vs. M0)	7.181	4.248-12.14	< 0.001	4.749	2.746-8.214	< 0.001
HVEM status (high vs. low)	2.053	1.225-3.439	0.006	1.998	1.178-3.390	0.010

HR, Hazard ratio; CI, confidence interval; Ra, rectum above the peritoneal reflection; Rb, rectum below the peritoneal reflection; C, cecum; RS, rectosigmoid; HVEM, herpesvirus entry mediator.

Furthermore, to our knowledge, the clinical involvement of HVEM in human CRC tissues has not been addressed. Based on a series of our previous studies and recent reports, we hypothesized that HVEM may be functionally important in human CRC (9, 17-19, 27-29). To clarify this possibility, we first investigated the potential role of HVEM expression in various colorectal lesions. Immunohistochemical analysis demonstrated that intense HVEM expression was not observed in normal colonic epithelium and hyperplastic polyps. In contrast, 24% of adenomas and more than half of all CRCs had high HVEM expression. These data suggested that HVEM expression might be involved in the development and progression of CRC. It is well-known that CRC develops *via* an adenoma to carcinoma sequence with the accumulation of a number of genetic and epigenetic mutations (30). Although at present it is unknown whether tumor HVEM expression is the cause or consequence of tumor progression in CRC, HVEM may play a role during the adenoma–carcinoma sequence.

Next, we evaluated the clinicopathological significance of tumor HVEM expression in CRC. We found that tumors with high HVEM expression had more advanced tumor status. In contrast, there were no significant correlations of tumor HVEM expression with nodal and metastatic status. These data suggested that HVEM might be involved in tumor progression rather than metastasis in CRC. Furthermore, the univariate prognostic analysis indicated that not only age, histopathological grading, tumor status, nodal status, and metastatic status, but also HVEM status had a significant prognostic value for patients with CRC. More importantly, multivariate analysis revealed that tumor HVEM expression was defined as an independent prognostic factor. Taken together, these data show that HVEM status may play a critical role in CRC independently of conventional TNM factors. We recently reported similar data on prognostic value in esophageal cancer (18). Therefore, the importance of

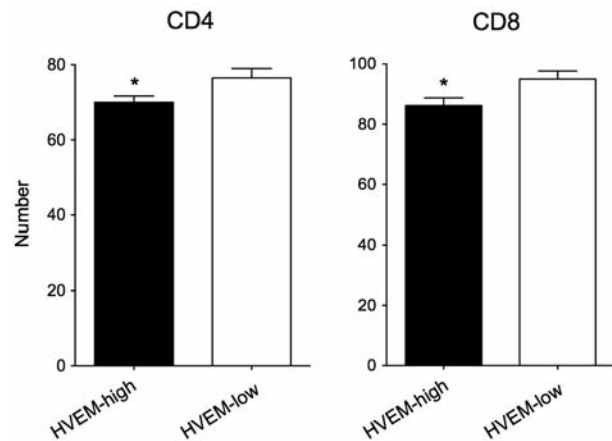


Figure 4. The number of tumor-infiltrating CD4⁺ and CD8⁺ lymphocytes were significantly lower in tumors with high herpesvirus entry mediator (HVEM) expression compared with those with low expression. **p* < 0.05.

HVEM may be a universal phenomenon in a variety of human malignant diseases. Further studies in other gastrointestinal and nongastrointestinal tumors are required to confirm this interpretation.

Finally, we investigated the underlying mechanism of involvement of tumor HVEM expression in CRC. We found that there was an inverse correlation between HVEM expression and the presence of TILs in CRC. The data suggested that HVEM might play a critical role in tumor immune evasion in human CRC. This is also consistent with our previous findings observed in esophageal cancer (18). Although TILs are thought to play a role in the inhibition of tumor progression and have prognostic significance in several human tumor types, including CRC, the precise underlying mechanisms are largely unknown (18, 21, 31,

32). Our data may highlight a potentially important role of HVEM in tumor immunity. Since other mechanisms, including cell proliferation and the cell cycle, may be involved in the role of HVEM in CRC, extensive basic studies are warranted in order to understand fundamental cancer biology and develop new therapeutic strategies.

Blocking negative regulatory pathways in T-cell activation has attracted much attention for cancer therapy. A randomized clinical trial has demonstrated that immunotherapy using antibody to CTLA4 improved overall survival in metastatic melanoma (25). Furthermore, both recent clinical trials and several basic studies have also suggested that targeting PD-L1/PD1 pathway holds promise for cancer treatment (26, 28, 29, 33). We and others have also reported that HVEM blockade enhanced tumor-reactive T-cell activation and inhibited tumor growth (18, 34). In fact, we have previously used a murine colon cancer model to investigate the *in vivo* efficacy of HVEM blocking and found that HVEM blockade exerted a significant antitumor effect (18). These data suggested that HVEM might be a novel target for cancer therapy against human CRC. However, HVEM blockade-alone may not be sufficient enough to induce significant clinical response especially for refractory tumors such as recurrent and metastatic CRC that acquired resistance to current conventional chemotherapy. Therefore, the combination of HVEM blockade with other anticancer strategies, including recent chemotherapy, anti-angiogenic and molecular-targeted therapies, that are currently used as the standard treatment for patients with CRC, may be desirable (3, 35).

In conclusion, we have demonstrated as far as we are aware of for the first time that HVEM may play a critical role in tumor progression and immune evasion in CRC. Our data also revealed that HVEM may be a novel prognostic marker and potential therapeutic target for human CRC.

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