Clinical impact of herpesvirus entry mediator expression in human hepatocellular carcinoma

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Abstract  Background: Herpes virus entry mediator (HVEM), also known as tumour necrosis factor receptor (TNFR) superfamily 14, regulates a variety of physiological and pathological responses in both innate and acquired immunity. Although HVEM is also suggested to be a critical regulator in tumours, actual roles in human cancer are largely unknown. This study aimed to clarify clinical importance of HVEM in human hepatocellular carcinoma (HCC).
Patients and methods: We studied HVEM expression in 150 HCC patients to explore its clinical relevance, and we examined tumour infiltrating T cells and local immune status of them.
Results: HVEM was expressed in HCC cells, while no or only limited expression was observed in normal tissues in the liver. Tumour HVEM expression was significantly correlated with age, serum protein induced by vitamin K absence or antagonist-II (PIVKA-II) level, vascular invasion and tumour node metastasis (TNM) stage. Furthermore, tumour HVEM expression significantly correlated with postoperative recurrence and survival. Importantly, multivariate analysis indicated that the HVEM status had an independent prognostic value. Furthermore, HVEM status was inversely correlated with tumour-infiltrating CD4+, CD8+ and CD45RO+ lymphocytes. In addition, it was also associated with reduced expression of perforin, granzyme B and interferon-γ (IFN-γ). Taken together, tumour-expressing HVEM plays a functionally important role in HCC.
Conclusion: Tumour-expressing HVEM plays a critical role in human HCC, possibly through regulating immune evasion. Therefore, targeting HVEM may be a novel promising therapeutic strategy for HCC.
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1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumours worldwide and the third cause of cancer-related death [1]. Despite improved diagnostic and treatment strategies, the postoperative prognosis of HCC remains unsatisfactory mostly due to frequent recurrence [2]. The treatments for HCC include surgical resection, radiofrequency ablation (RFA), transcatheter arterial chemoembolisation (TACE) and radiation. Furthermore, multi-tyrosine kinase inhibitor has recently been approved as a new therapeutic option for advanced HCC [3]. However, none of them is enough to completely prevent tumour formation and recurrence. Clearly, new therapeutic strategies are needed to improve the survival of patients with HCC. One of potential promising strategies may be immunotherapy. In fact, it has been reported that there are a number of tumour-infiltrating T cells in HCC tissue [4,5]. Furthermore, recent studies have revealed that some specific subsets of T cells may be functionally important and also have prognostic value [6,7]. Importantly, a randomised clinical trial has shown that certain adoptive immunotherapy has significantly inhibited postsurgical recurrence in humans [8]. However, on the other hand, clinical efficacy of immunotherapy is very limited to date [9]. This may be, at least in part, due to tumour immune evasion through a variety of mechanisms [10–13].

Herpes virus entry mediator (HVEM), also known as tumour necrosis factor receptor (TNFR) superfamily 14 (TNFRSF14), was identified as a cellular mediator of herpes simplex virus entry [14]. It is expressed on several types of cells, including T cells, B cells, NK cells, dendritic cells, and myeloid cells, as well as non-lymphoid organs including lung, liver and kidney [14,15]. HVEM is a ligand for the immunoglobulin (Ig) superfamily members B-lymphocyte and T-lymphocyte attenuator (BTLA) and CD160, and is also a receptor for the TNF superfamily members LIGHT and LTα [16,17]. HVEM expressing on haematopoietic cells is known to display a dual functional activity for both T-cell activation and inhibition depending on the ligands engaged [16–18]. Previous studies have reported the roles of HVEM pathway in several disease conditions including autoimmune disease, infection and inflammation [18,19]. In contrast, there are relatively few reports on the roles of HVEM in tumours. HVEM on tumour cells has been shown to inhibit cytokine production and proliferation of tumour antigen-specific CD8+ T cells via BTLA in vitro [20]. Furthermore, several murine studies have demonstrated that blockade of HVEM/BTLA pathway augments tumour antigen-specific immune responses and inhibits tumour growth [21,22]. Others have also reported that LIGHT mediates tumour cell apoptosis via signalling through tumour-expressed HVEM, leading to the suppression of tumour growth [23,24]. Taken together, the functions of HVEM in tumour environments are likely to be immunosuppressive rather than activating tumour immunity. However, to date, there are very few clinical studies on the HVEM expression on human malignant tumours. Although a few studies have shown HVEM expression and gene polymorphisms in actual human malignancies such as melanoma, breast cancer and haematopoietic cancer, the precise functions of HVEM in human tumours are still largely unknown [20,24–26].

The aim of this study was to clarify clinical significance of HVEM in human HCC. Furthermore, in order to reveal immunological function of HVEM, we investigated the correlation of HVEM expression with several subsets of tumour-infiltrating T cells and local immune status.

2. Patients and methods

2.1. Patients and specimens

We examined 150 patients with HCC who underwent surgery at the Department of Surgery, Nara Medical University, between 2000 and 2012. Follow-up was until death or September 2013. The median follow-up for all patients was 51.8 months, with a range of 6.7–151.7 months. None of the patients received anticancer therapy before surgical resection. Clinicopathological stages were classified according to the International Union against Cancer. For in vitro experiments, 49 fresh tissues out of the above 150 resected specimens were rapidly frozen at −80 °C for storage until use. The remainder of each specimen was fixed in 10% phosphate-buffered formalin, embedded in paraffin. Written informed consent was obtained from all patients before treatment, according to our institutional guidelines. This study was approved by the institutional review board.

2.2. Immunohistochemistry

Formalin-fixed, paraffin-embedded tissues of primary tumour were cut into 5-μm sections, deparaffinised and rehydrated in a graded series of ethanol. Antigen retrieval was carried out by heating tissue sections using a Target Retrieval Solution, pH 9.0 (DAKO, Tokyo, Japan). To block endogenous peroxidase, sections were immersed in 3% solution of hydrogen peroxide in absolute methanol for 5 min at room temperature and washed thrice in fresh PBS, each of 5 min duration. Then the sections were incubated overnight at 4 °C with anti-human HVEM/TNFRSF14 antibody (MAB3561, monoclonal mouse, R&D Systems) diluted 1:20 with Antibody Diluent (DAKO) or anti-human CD45RO (UHL1, monoclonal mouse; DAKO), CD4 (1:40) (4B12, monoclonal mouse; DAKO) and CD8 (C8/144B, monoclonal mouse; DAKO). Sections were washed thrice in PBS, fol-
lowed by incubation with the EnVision detection system (DAKO), according to the instructions of the manufacturer. Sections were counterstained with haematoxylin, dehydrated in ethanol, cleared in xylene and coverslipped.

2.3. Evaluation of immunostaining

Immunohistochemistry for HVEM was evaluated by authorised pathologists who had no knowledge of the patients’ clinical status and outcome. At least 1000 tumour cells were scored, and percentage of tumour cells showing positive staining was calculated, as previously reported [26]. Tumour-infiltrating CD45RO⁺, CD4⁺ and CD8⁺ T cells into HCC tissues were evaluated [27]. Each sample was thoroughly evaluated at a magnification of ×200. To count stained cells, five randomly selected areas were counted independently by two investigators without knowledge of clinical information. The median value was calculated for each sample.

2.4. Extraction of total RNAs and real-time reverse transcriptase polymerase chain reaction (PCR) analysis

Total RNA was isolated using RNAspin Mini (GE Healthcare, Tokyo, Japan) and the first-strand cDNA was synthesised from 1 μg RNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, United States of America (USA)), according to the instructions of the manufacturer. Real-time quantitative PCR analysis was carried out using an ABI Prism 7700 sequence detector system (Applied Biosystems). All primer/probe sets were purchased from Applied Biosystems. PCR was carried out using the TaqMan Universal PCR Master Mix (Applied Biosystems) using 1 μl of cDNA in a 20 μl final reaction volume. The PCR thermal cycle conditions were as follows: initial step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The expression level of the housekeeping gene β2-microglobulin was measured as an internal reference with a standard curve to determine the integrity of template RNA for all specimens. The ratio of the mRNA level of each gene was calculated as follows: (absolute copy number of each gene)/(absolute copy number of β2-microglobulin) [26].

2.5. Statistical analysis

Age, serum AFP and PIVKA-II protein induced by vitamin K absence or antagonist-II (PIVKA-II) levels were dichotomised at the median. Statistical significance between two groups of parametric date was evaluated using the Student’s t test or the chi-square test as appropriate. Survival curves were estimated using the Kaplan–Meier method, and the significances of differences between survival curves were determined using log-rank test. Multivariate comparisons of survival distributions were made using Cox proportional hazards models. A value of $p < 0.05$ was considered statistically significant in all analyses.

3. Results

3.1. Tumour HVEM expression in human hepatocellular carcinoma

We first examined the expression of HVEM in 150 surgically resected HCC tissues by immunohistochemistry. The expression of HVEM was observed in the cell membrane, cytoplasm, or both of hepatocellular carcinoma cells (Fig. 1). On the other hand, there was no or only limited expression of HVEM in other normal tissues in the liver including normal hepatocyte, sinusoidal cell, bile duct epithelial cell and vascular endothelium.

3.2. Correlation between tumour HVEM expression and clinicopathological characteristics

To investigate the clinical importance of tumour HVEM expression in HCC, we divided 150 cases into high HVEM group (≥50% of HVEM-positive tumour cells) and low HVEM group (<50% of HVEM-positive tumour cells).

Fig. 1. Representative case of high and low expression of herpes virus entry mediator (HVEM) in surgically resected hepatocellular carcinoma (HCC) tissues. (A) High HVEM expression case (>50% of HCC cells). (B) Low HVEM expression case (<50% of HCC cells).
cells) and low HVEM group (<50% of HVEM-positive tumour cells). Then, we examined correlation between HVEM expression and various clinicopathological characteristics (Table 1). As a result, HVEM expression was significantly correlated with age ($P = 0.024$), presence of vascular invasion ($P = 0.022$), serum PIVKA-II level ($P = 0.009$) and tumour node metastasis (TNM) stage ($P = 0.036$).

3.3. Impact of tumour HVEM expression on postoperative recurrence and survival

Next, we compared postoperative recurrence and survival according to HVEM status (Fig. 1). As a result, high HVEM group had significantly poorer recurrence-free survival (RFS) and overall survival (OS) than low HVEM group (Fig. 2). The median RFS time was 1.3 and 4.9 years, respectively ($P < 0.001$). Moreover, the median OS time was 4.8 and 8.5 years, respectively ($P = 0.002$). Five-year postoperative survival rate of high-HVEM and low-HVEM patients was 47.9% and 78.4%, respectively.

3.4. Prognostic value of tumour HVEM expression in HCC

Furthermore, we examined the prognostic value of tumour HVEM expression in HCC. In univariate analysis, serum PIVKA-II level, tumour size, vascular invasion, TNM stage and tumour HVEM expression were significantly associated with both RFS and OS (Table 2). Multivariate analysis revealed that tumour size ($P = 0.028$) and tumour HVEM expression ($P < 0.001$) were significant factors for RFS (Table 3). In terms of OS, tumour size ($P = 0.003$) and tumour HVEM expression ($P = 0.008$) were turned out to be statistically significant (Table 3).

3.5. Correlation between tumour HVEM expression and recurrence pattern

In total, 86 recurrences (57.3%) were confirmed until the final follow-up. While intrahepatic recurrence was observed in 78 patients, extrahepatic recurrence was found in 36 patients. The sites of extrahepatic recurrence were the lung in 23, the bone in 12 and others in six. There were significant correlations of HVEM expression with both intrahepatic and extrahepatic recurrence (Table 4). In particular, early recurrence within 2 years after surgery was found more frequently in high HVEM group.

3.6. Association of HVEM expression with tumour-infiltrating T cells and local immune status

To investigate the underlying mechanism of prognostic impact of tumour HVEM expression in HCC, we examined tumour-infiltrating T cells by immunohistochemistry. As a result, we found that the numbers of tumour infiltrating CD4$^+$, CD8$^+$ and CD45RO$^+$ T cells were significantly lower in high HVEM group compared to low HVEM group (Fig. 3). These data suggested that tumour HVEM expression might prevent the infiltration of T cells into HCC tissues. Furthermore, to evaluate local immune status, we examined the expression of perforin, granzyme B and interferon-γ (IFN-γ) in HCC tissue by real time PCR analysis on available 49 frozen tissues. The expressions of perforin, granzyme B as well as IFN-γ were significantly lower in high HVEM group than low HVEM group (Fig. 4). Data indicated that tumour HVEM expression might be correlated with the inhibition of local immune activation. Taken together, HVEM may play a functionally important role through regulating the infiltrations of T cells into HCC.

4. Discussion

HVEM has been shown to possess diverse and unique immunological functions in various physiological and pathological conditions [28]. One of clinically relevant functions of HVEM may be involvement in tumour immune evasion. We have recently reported that HVEM plays a critical role in the evasion of host antitumour immune responses in human oesophageal cancer,
suggesting that it may be promising therapeutic target [26]. Consistent with our previous report on oesophageal cancer, this study has also shown that there are inverse correlations between tumour-infiltrating T cells and tumour HVEM expression. We also confirmed the local immune suppression of HCC tissue in association with HVEM expression. More importantly, this study revealed that tumour HVEM expression correlated with postoperative recurrence and also had an independent prognostic value for HCC. To date, although several studies have suggested the potential clinical importance and efficacy of immunotherapy in HCC, fundamental immunological mechanisms are largely unknown in tumour biology of HCC [4–8,29,30]. Our previous study has shown that PD-L1, one of potent immune checkpoint inhibitors, might be a predictor of recurrence for HCC patients [13]. This study further suggested that immune evasion through negative regulatory mechanisms, including HVEM and PD-L1, plays a pivotal role in HCC patients. Taken together, immunotherapy

Table 2
Univariate analysis of factors associated with recurrence-free and overall survival.

<table>
<thead>
<tr>
<th>Number</th>
<th>Recurrence free survival</th>
<th>Overall survival (OS)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Hazard ratio (HR)</td>
<td>95% Confidence interval (CI)</td>
</tr>
<tr>
<td>Age &lt;70/≥70</td>
<td>77/73</td>
<td>1.18</td>
</tr>
<tr>
<td>Gender Male/Female</td>
<td>116/34</td>
<td>0.76</td>
</tr>
<tr>
<td>Cirrhosis Absent/Present</td>
<td>93/57</td>
<td>0.77</td>
</tr>
<tr>
<td>Viral status Non/Hep B + C</td>
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</tr>
<tr>
<td>AFP, ng/ml &lt;15/≥15</td>
<td>72/78</td>
<td>1.32</td>
</tr>
<tr>
<td>PIVKA-II, U/ml &lt;150/≥150</td>
<td>77/73</td>
<td>1.85</td>
</tr>
<tr>
<td>Tumour size, cm &lt;5/≥5</td>
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<td>2.37</td>
</tr>
<tr>
<td>Tumour number Single/Multiple</td>
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<td>1.69</td>
</tr>
<tr>
<td>Histological differentiation Well/Mod, Por</td>
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<td>1.22</td>
</tr>
<tr>
<td>Vascular invasion Absent/Present</td>
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<td>1.59</td>
</tr>
<tr>
<td>Capsule invasion Absent/Present</td>
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<td>1.18</td>
</tr>
<tr>
<td>TNM stage I/II–IV</td>
<td>69/81</td>
<td>1.58</td>
</tr>
<tr>
<td>Herpes virus entry mediator (HVEM) expression Low/High</td>
<td>84/66</td>
<td>2.77</td>
</tr>
</tbody>
</table>

Table 3
Multivariate analysis for the predictors of recurrence-free and overall survival.

<table>
<thead>
<tr>
<th>Recurrence-free survival</th>
<th>Overall survival (OS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hazard ratio (HR)</td>
<td>95% Confidence interval (CI)</td>
</tr>
<tr>
<td>PIVKA-II, U/ml &lt;150/≥150</td>
<td>1.20</td>
</tr>
<tr>
<td>Tumour size, cm &lt;5/≥5</td>
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</tr>
<tr>
<td>Vascular invasion Absent/Present</td>
<td>1.18</td>
</tr>
<tr>
<td>Herpes virus entry mediator (HVEM) expression Low/High</td>
<td>2.54</td>
</tr>
</tbody>
</table>
targeting immune checkpoints may hold a great promise for improving prognosis of HCC patients.

Most cases of HCC are secondary to either a viral hepatitis infection or alcoholic cirrhosis. Interestingly, recent studies have reported that HVEM-BTLA signaling is likely to affect the pathogenesis of HBV-related acute-on-chronic liver failure [31]. Furthermore, HVEM/TNFRSF14 gene expression was also shown to be upregulated in human alcoholic hepatitis [32]. These studies have suggested that HVEM might play

### Table 4
Correlation between tumour herpes virus entry mediator (HVEM) expression and recurrence.

<table>
<thead>
<tr>
<th></th>
<th>High herpes virus entry mediator (HVEM)</th>
<th>Low HVEM</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intrahepatic recurrence</td>
<td>Present 42</td>
<td>36</td>
<td>0.012</td>
</tr>
<tr>
<td></td>
<td>Absent 24</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>Extrahepatic recurrence</td>
<td>Present 22</td>
<td>14</td>
<td>0.018</td>
</tr>
<tr>
<td></td>
<td>Absent 44</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>Early recurrence within 2 years</td>
<td>Present 35</td>
<td>16</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Absent 31</td>
<td>68</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 3. Correlation between tumour herpes virus entry mediator (HVEM) expression and tumour-infiltrating lymphocytes. Representative images of immunohistochemical staining of tumour-infiltrating T cell subsets are shown on the left. The number of tumour-infiltrating CD4+, CD8+ and CD45RO+ lymphocytes was significantly fewer in HVEM-high tumours compared with HVEM-low tumours (P = 0.002, P = 0.001 and P = 0.001, respectively). (mean ± SEM (the standard error of the mean)).
some roles in pathogenesis of hepatitis related to either viral infection or alcohol use. Postoperative recurrence of HCC has been suggested to be associated with accompanying active hepatitis [33]. Therefore, the treatment of hepatitis after surgery for HCC may lead to the inhibition of postoperative recurrence and improvement of patient survival. However, to date, several clinical trials to evaluate interferon against hepatitis virus have failed to show reliable efficacy and further suggested the need for more potent antiviral therapy [33,34]. Although neither viral status nor cirrhosis correlated with tumour HVEM expression in this study, targeting HVEM expressed in the liver may lead to not only direct antitumour effect but also the inhibition of active hepatitis. Since our data indicated that HVEM expression was significantly associated with early postoperative recurrence, further studies may be warranted.

A major challenge in HCC treatment is to prevent postoperative recurrence. However, since most current treatments for HCC, including surgery, RFA, TACE and radiation, are theoretically local control of tumours, those are basically ineffective for the prevention of multicentric occurrence of HCC. Immunotherapy may be expected to induce systemic rather than local antitumour effect. In fact, CTLA-4 or PD-1 blockade has been evaluated in clinical trials, and demonstrated to induce significant response and prolong patient survival [35,36]. Our data warrant further study to evaluate clinical efficacy of targeting HVEM as a new immunological checkpoint inhibitor in human malignancies. Especially for HCC, HVEM blockade may be desirable, since it has a dual effect through inhibiting tumours as well as active hepatitis as described above. In addition, we and others have reported that memory T cells expressing CD45RO may be more important for patient survival than other subsets of T cells in various cancer [37–39]. Our data indicated that tumour HVEM expression was inversely correlated with not only CD4+ or CD8+ T cells but also CD45RO+ T cells in HCC. Therefore, HVEM blockade may lead to promote the infiltrations of memory T cells into the tumour, thereby resulting in long-lasting antitumour efficacy in HCC patients.

In clinical setting, the combination therapy of HVEM blockade with some other anticancer treatments including chemotherapy, radiotherapy, antiangiogenesis and blockade of other immunocheck point including PD-1 and CTLA-4 may induce synergistic anticancer effects. In fact, we have shown synergistic effects induced by several combinations of blocking T cell negative pathways with other strategies such as chemotherapy and antiangiogenic treatment [11,12,40]. However, careful evaluation by clinical trials is needed, since blockade of immunocheck point may induce unfavorable effect such as autoimmune diseases.

There are several limitations in this study. The number of samples evaluated for this study is relatively small. In addition, this study was conducted in a single institution. Therefore, a large-scale and multicenter international collaborative study for HVEM is critically important to confirm reproducibility of our data. Furthermore, to our best of knowledge, there is no antibody available for human HVEM blockade in clinical trial. Therefore, further studies not only to generate new antibody but also to clarify its action in vivo are needed, since the antibodies to inhibitory receptor pathways may not be always act as expected.

In conclusion, we have shown for the first time that tumour HVEM expression may be associated with the development of HCC. Furthermore, it is inversely correlated with tumour infiltrating T cells and local immune...
activation, suggesting that HVEM plays a functionally important role in HCC. Therefore, targeting HVEM may be a novel promising therapeutic strategy for HCC.

Conflict of interest statement
None declared.

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