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The immune checkpoint, HVEM may contribute to immune escape in non-small cell lung cancer lacking PD-L1 expression

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ABSTRACT

Background: : Herpes Virus Entry Mediator (HVEM) is an important immune checkpoint in cancer recognition. HVEM expressed on tumor cell membranes activates immune cell signaling pathways leading to either inhibition of activity (B- and T-lymphocyte attenuator, BTLA) or activation of immune activity (LIGHT). The aim of this study is to investigate the prevalence of HVEM expression and its association with PDL1 expression in NSCLC.
Methods: : A TMA of 527 resected NSCLC samples and 56 NSCLC cell lines were evaluated for HVEM and PD-L1 expression. The IHC assay for HVEM was optimized on the Dako Link48 autostainer using a polyclonal antibody from R&D Systems(AF356). PD-L1 IHC was performed on the Dako Link48 autostainer using the PD-L1 28-8 pharmDx kit. Scoring HVEM employed the H-score system while for PD-L1 the tumor proportion score (TPS) was used.
Results: : HVEM expression in the NSCLC resected samples and cell lines revealed a positive H-score more than 1 was 18.6% (77/415) and 48.2% (27/56) respectively. HVEM expression was significantly higher in patients with lymph node N2 metastasis (25.5% vs 7.9% vs 17.5%, $p = 0.046$) when comparing with N1 or no lymph node metastasis, and was marginally significantly higher in patients with stage III/IV disease (24.5% vs 16.4%, $p = 0.059$). Subgroup analysis showed that HVEM (median 45 vs 36 months, $p = 0.706$) and PD-L1 expression (median 45 vs 48 months, $p = 0.178$) status was not predictive of overall survival. HVEM was found to have a significant negative correlation with PD-L1 expression ($r = -0.274$, $p < 0.001$) in patients with NSCLC and also a weak negative correlation in NSCLC cell lines ($r = -0.162$, $p = 0.352$).
Conclusion: : HVEM was found to be overexpressed in NSCLC patients of N2 lymph node metastasis or later stage and has a negative co-relationship with PD-L1 expression. HVEM was not prognostic for NSCLC patients.

1. Introduction

The success of anti-PD1/PDL1 immunotherapy has ushered in a new pillar of therapy for non-small cell lung cancer (NSCLC) patients. Several landmark clinical trials have demonstrated superior efficacy of anti-programmed death-1 (PD-1) or programmed death-ligand 1 (PD-L1) monoclonal-antibodies, including nivolumab, pembrolizumab and atezolizumab, compared with docetaxel as second-line treatment for patients with advanced NSCLC [1–4]. Additionally, pembrolizumab has been approved by the Food and Drug Administration (FDA) in the first-line setting in EGFR/ALK wild type NSCLC with a PD-L1 tumor proportion score (TPS) ≥ 50% due to a significant improvement in progression-free survival (PFS) and overall survival (OS) [5,6]. However, majority of patients did not respond to anti-PD1/PDL1 immunotherapy, which suggests NSCLC tumors may escape immune surveillance from other mechanisms, for example, involvement of other checkpoint

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inhibitors such as TIM3 and Lag3 may enhance efficacy of immunotherapy when combined with anti-PD1/PDL1 therapy [7].

Herpes virus entry mediator (HVEM), also called tumor necrosis factor receptor superfamily member 14 (TNFRSF14), is another checkpoint target and could interact with both TNFRSFs (LIGHT, lymphotoxin-like, exhibits inducible expression, and competes with herpes simplex virus glycoprotein D for HVEM, a receptor expressed by T lymphocytes, and lymphotoxin-a) and immunoglobulin (Ig) superfamily members [B and T lymphocyte attenuator (BTLA) and CD160] [8]. HVEM was initially found to be highly expressed in melanoma cell lines [9] and has recently been detected in other cancers, including colorectal, liver, breast and esophageal cancers [10–13]. HVEM was found to be correlated with poor patient survival and advanced cancer features [10–13]. In addition, HVEM gene silencing was able to inhibit cell proliferation and induce cell cycle arrest in human esophageal carcinoma cells [12]. However, the role and clinical relevance of HVEM remains largely unknown in lung cancer.

In order to investigate the prognostic role of HVEM and its association with clinical-pathological features or PDL1 expression in NSCLC, we evaluated a cohort of 527 patients and found HVEM was overexpressed in patients with advanced disease or lymph node metastasis and has a negative co-relationship with PD-L1 expression, HVEM did not have a prognostic role. We also validated the expression of HVEM and association with PDL1 expression in a cohort of 56 NSCLC cell lines.

2. Methods

2.1. Patients

Primary tumor specimens were obtained from 527 surgical patients with NSCLC at Olivia Newton-John Cancer and Wellness Centre, Austin Hospital, Australia, from Jan 1992 to Oct 2010 with patient consent. The patients had not undergone radiotherapy or chemotherapy before surgery. The surgical histology reports were reviewed and the lymph node and lung cancer stages were categorized according to the seventh edition of the International Association for the Study of Lung Cancer TNM staging system. The study was approved by the Austin Hospital's Institution Review Board.

2.2. Cell lines

56 characterized NSCLC cell lines in the Hirsch Laboratory culture collection were grown to confluence in RPMI 1640 media with 10% bovine serum and 1X pen/strep. Cells were harvested, fixed overnight in formalin, centrifuged to a soft pellet, mixed with 0.9% agarose, and allowed to solidify at room temperature to a pellet. Each cell line pellet was placed in a cassette and transferred to 70% ethyl alcohol, processed and embedded in paraffin blocks. Cores (1 mm) were taken from each cell line block to create the TMA from which 4-μm sections were cut and stained.

2.3. HVEM IHC staining

An IHC assay was optimized for HVEM using a commercial antibody from R&D Systems (AF356, polyclonal Goat IgG). Positive controls included spleen and tonsil tissue while negative controls included adipose tissue and brain cortex. The reconstituted antibody was diluted 1:200 with Signal Stain antibody diluent (#8112, Cell Signaling). Staining was performed on a Dako Link 48 using the Flex pH6 target retrieval on the PT-Link processor. Secondary antibody was the Vecta Stain Elite AVC kit (PK6105, Vector) and DAB reagent was the Dako DAB/Chormagen (K3468/K1494). Stained slides were run down in ethanol/xylene followed by automated coverslipping on a TissueTek 4795.

2.4. PD-L1 IHC staining

Sections were stained for PD-L1 using the Dako Link 48 autostainer and the Dako 28-8 pharmDx kit as per the manufacturers protocol. A positive and negative control slide provided by Dako was augmented with tonsil tissue and provided an in-run control. Stained slides were run down in ethanol/xylene followed by automated coverslipping on a TissueTek 4795.

2.5. IHC scoring

All IHC scoring was performed by the research pathologist in the Hirsch Lab (HY) with inter-observer scoring performed by an outside pathologist. HVEM stained specimens were scored using the H-score system while PD-L1 staining was scored using the Tumor Proportion Score (TPS) system. For discrepant results, a final score was determined by a consensus conference of the pathologists. Scores for specimens with multiple cores were averaged. Scoring details are further described in previous studies [14,15].

2.6. Western blot

Cell lines were grown to confluence in 10 cm culture dishes, washed 3x with cold PBS and scraped with 1x cell lysis buffer (#9803, Cell Signaling) with AEBSF (A8456, Sigma). Following sonication on ice with a pencil-type sonicator (Sonifier 450, Branson), cells were subjected to a freeze-thaw cycle, vortexed for 30 s and centrifuged at 4 °C at 10,000 × *g* for 20 min. Soluble protein was removed and assayed using the BCA Protein kit (Pierce). Western blots were performed using precast Criterion 4–20% gradient gels (Bio-Rad). All wells were loaded with 25 μg of total protein. Following protein transfer to PVDF membrane, the blot was blocked in 5% milk in TTBS for 1 h then probed with the anti-HVEM antibody diluted 1:1000 overnight at 4 °C with rocking. Following 5 washes with TTBS, the blot was incubated in mouse anti-goat HRP antibody (sc-2354, Santa Cruz). The blot was revealed using Clarity Western ECL Substrate (170–5060, Bio-Rad) and recorded on high contrast autoradiography film. Western blots for PD-L1 were performed with the same protocol but using the anti-PD-L1 antibody 28-8 (ab205921, Abcam) and an anti-rabbit HRP linked secondary antibody (#7074, Cell Signaling).

2.7. Statistical analysis

The findings were analyzed using SPSS statistical software (version 23.0; SPSS, Chicago, IL). Chi square analysis or Fisher's exact test were used to test for the association of HVEM expression with Clinical-pathologic features, while the pearson correlation coefficient test was used for analyzing the association of HVEM expression and PDL1 expression in both the clinical cohort and cell line cohort. The pearson coefficient ranges from -1 to 1. A value of 0 implies that there is no linear correlation between the variables. A value over 0 implies the positive correlation that dependent variables increase as independent variables increase. A value less than 0 implies the negative correlation that dependent variables decrease as dependent variables increase. Survival data were evaluated using Kaplan–Meier analysis. All statistical tests were two-sided, and statistical significance was defined as $p \leq 0.05$.

3. Results

3.1. Patient characteristics

The clinical-pathologic features of the Mitchell cohort are described in Table 1. A total of 527 patients were included in the analysis. The age of the patients ranged from 29 to 85 years (mean 65.7). There were 228 (43.3%), 172 (32.6%), 119 (22.6%) and 8(1.5%) patients with stage I,