Clinical Cancer Research

HHLA2, a New Immune Checkpoint Member of the B7 Family, Is Widely Expressed in Human Lung Cancer and Associated with EGFR Mutational Status

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Abstract

Purpose: Immunotherapy with antibodies against B7/CD28 family members, including PD-1, PD-L1, and CTLA-4 has shifted the treatment paradigm for non–small cell lung carcinoma (NSCLC) with improved clinical outcome. HHLA2 is a newly discovered member of the family. By regulating T-cell function, HHLA2 could contribute to tumor immune suppression and thus be a novel target for cancer immunotherapy. There is limited information and critical need to characterize its expression profile and clinical significance in NSCLC.

Experimental Design: We performed IHC with an HHLA2-specific antibody (clone 566.1) using tissue microarrays constructed from 679 NSCLC tumor tissues, including 392 cases in the discovery set and 287 cases in the validation cohort. We also studied clinicopathologic characteristics of these patients.

Results: Overall, HHLA2 was not detected in most of normal lung tissue but expressed in 66% of NSCLC across different subtypes. In particular, *EGFR*-mutated NSCLC was significantly associated with higher tumor HHLA2 expression in both discovery (*EGFR* vs. WT: 76% vs. 53%, $P = 0.01$) and validation cohorts (89% vs. 69%, $P = 0.01$). In one of the two cohorts, HHLA2 expression was higher in lung adenocarcinoma as compared with squamous and large cell histology, non-Hispanic White versus Hispanics, and tumors with high tumor-infiltrating lymphocyte (TIL) density. In the multivariate analysis, *EGFR* mutation status and high TIL intensity were independently associated with HHLA2 expression in lung adenocarcinoma.

Conclusions: HHLA2 is widely expressed in NSCLC and is associated with *EGFR* mutation and high TILs in lung adenocarcinoma. It is potentially a novel target for lung cancer immunotherapy. *Clin Cancer Res; 23(3); 825–32. ©2016 AACR.*

Introduction

Lung cancer is the most common cause of cancer-related mortality in the United States, accounting for more deaths than breast, prostate, and colorectal cancers combined in 2015 (1). The treatment for non–small cell lung cancer (NSCLC) has substantially changed over the past decade with the identification of driver mutations and the recent approval of immune checkpoint inhibitors. State-of-the-art treatment paradigms now incorporate histology-driven approaches (2) and targeted treatment of molecularly defined subsets in lung cancer (3). The ability to evade the immune system is considered one of the hallmarks of cancer (4, 5). One of the mechanisms of immune evasion in lung cancer is the expression of immune checkpoints on tumor cells. When an antigen is presented through the MHC complex, a simultaneous signal is required which determines the type of T-cell response; a costimulatory signal causing activation of the T cell while a coinhibitory signal results in T-cell inhibition. When tumor cells express coinhibitory ligands, this results in T-cell exhaustion and subsequently a blunted immune response which represents one of the mechanisms of tumor immune evasion (6–8).

The CD28 family of receptors and the B7 family of ligands provide the T-cell costimulatory and coinhibitory signals (6, 9). The well-established members of the CD28 receptor family include CD28, CTLA-4, ICOS, and PD-1, whereas the B7 ligands for these corresponding receptors are B7-1, B7-2, ICOS-L, PD-L1, and PD-L2. Other B7 ligands have been discovered in the past decade namely B7-H3 (CD276; ref. 10) and B7x (B7-H4/B7S1; refs. 11–13) and have been shown to suppress tumor immune response when expressed in various cancers (6, 8, 14–19). HHLA2 is the most recently discovered ligand of the B7 family (20–23), and was first demonstrated as a T-cell coinhibitory molecule by us (20, 24). Blocking immune checkpoints has become an important

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doi: 10.1158/1078-0432.CCR-15-3071

Translational Relevance

New immunotherapies of immune checkpoint inhibitors targeting members of the B7 and CD28 families, such as PD-1 and PD-L1, have recently been exploited with therapeutic benefits in human lung cancer. HHLA2 is a newly discovered B7 family member with T-cell coinhibitory function. Here, we show that HHLA2 protein is not detected in most of normal lung tissue but overexpressed in human lung cancer. Furthermore, HHLA2 expression is independently associated with *EGFR* mutation and high TIL infiltration in lung adenocarcinoma. This is the first study reporting the clinical significance of HHLA2 in lung cancer, and suggests that HHLA2 might be a novel immunosuppressive mechanism within the lung tumor microenvironment as well as a new target for lung cancer immunotherapy.

treatment option in the management of NSCLC and inhibition of the PD-1/PD-L1 pathway has been shown to have a therapeutic benefit (7, 25). The FDA has approved PD-1 inhibitors, nivolumab and pembrolizumab, as second-line treatments for advanced NSCLC, because they have been shown to improve overall survival as compared with conventional second-line chemotherapy (26, 27). Hence, this is a therapeutic approach of great interest and it is important to explore other immune checkpoints which could contribute to immune evasion, apart from PD-1/PD-L1. The expression and clinical significance of the newest immune checkpoint, HHLA2, in human cancer is unclear. In this report, we hereby explored its expression and its association with clinical outcome in human NSCLC.

Materials and Methods

Patients and samples

Tissue microarrays (TMA) in the discovery cohort were constructed from 392 NSCLC tumor tissues and the TMAs in the separate validation cohort were consisted of 287 NSCLC cases (mostly derived from stage I to III NSCLC specimens from patients undergoing lung cancer resection). The TMAs used 1.0-mm cores with all cases in triplicates. Areas of tumor were selected by one of the coauthors (A. Borczuk; a dedicated pulmonary pathologist), based on correlation with corresponding slide to identify tumor-containing areas. In the triplicate sampling, three different areas of the tumor were sampled, with at least one of the three cores at the periphery of the nodule/mass, but still containing tumor.

The relevant clinical data and mutational status were collected through retrospective chart reviews. Race of a patient was based on self-identification. All protocols were reviewed and approved by the Institutional Review Board.

IHC

As described previously (28), formalin-fixed primary lung tumor tissue sections were deparaffinized followed by antigen retrieval treatment with sodium citrate (10 mmol/L, pH 6.0). Endogenous peroxidase activity was blocked by Dako peroxidase blocking reagent (Dako Corporation). A mouse anti-human HHLA2 mAb (clone 566.1, IgG1; refs. 20, 24) was used at a concentration of 4 μg/mL (dilution of 1:500 for overnight incubation) at room temperature. Then Dako Envision system-horseradish peroxidase (HRP; Dako Corporation) was used by adding biotinylated link universal and streptavidin-HRP followed by DAB chromogen (Dako Corporation) and hematoxylin nuclear counterstaining. The mAb 566.1 specifically recognizes human HHLA2 but not other human B7 family members (B7-1, B7-2, ICOS-L, PD-L1, PD-L2, B7-H3, and B7x) or human CD28 family members (CD28, CTLA-4, ICOS, PD-1, and TMIGD2; ref. 20). As reported previously (29), FFPE specimens in which 3T3 cells were expressing HHLA2 or CTLA-4 served as positive and negative controls, respectively.

The expression of HHLA2 was evaluated by using IHC in FFPE patients' specimens by two independent investigators and its immunohistochemical semiquantitation was performed using the H-score. On the basis of the intensity of staining HHLA2 expression was quantified as 0, 1, 2, and 3 for absent, low, moderate, and high expression. The H-score was determined as the percentage of staining (proportion score) multiplied by an ordinal value corresponding to the maximum intensity score in the specimen (0 = none, 1 = weak, 2 = moderate, 3 = high).

Assessment of tumor-infiltrating lymphocytes

As described previously (30), the scoring of tumor-infiltrating lymphocyte (TIL) was performed in the same slides used for HHLA2 staining with hematoxylin and eosin (H&E). Slightly modified from the published method (30), the TIL system was read using a three-tiered scale based on the visual estimation of the proportion of lymphocytic infiltration in each histospot. A score of "Absent" indicated absence of TILs, "Low" was defined as 1% to 30%, and "High" was defined as >30% (Fig. 1A).

Statistical analysis

For categorical variables after checking for assumptions, χ^2 or Fisher exact test were appropriately used to compare the distribution of each categorical variables between HHLA2 expression positive and negative groups. For pairwise comparisons, two-sample proportion test was performed to test whether the frequency of positive HHLA2 expression is greater in one mutational, histologic, or ethnic subgroup than another. Wilcoxon rank sum test was performed to test whether the median HHLA2 H-score was greater in one mutational, histologic, ethnic, or TIL subgroup than another. Logistic regression models were used for multivariate analysis. For survival analysis, comparison of the Kaplan–Meier survival curves between HHLA2 expression positive and negative groups or different degree of TIL groups were performed using log-rank test. All tests were two-sided using the SAS 9.2 software (SAS Institute Inc.). All P values ≤ 0.05 were considered significant.

Results

Clinical and pathologic features of patients and tumors

The majority of samples from the TMA sets are well annotated. In the discovery cohort ($n = 392$ patients), the mean age of the patient population was 67.6 years. Among them, 85% (334/392) had stage I–III NSCLC disease and underwent surgical resection from 2000 to 2012 whereas 0.5% (2/392) patients had stage IV disease. Race was documented for 280 patients, including 82% (230/280) of non-Hispanic White, 9% (25/280) of Hispanic, 6.4% (18/280) of African American, and 1.2% (7/280) of Asian patients. Histology was available for all the cases: the cohort consisted of 74% (290/392) lung adenocarcinoma, 8%

(31/392) squamous, and 4.1% (16/392) large-cell NSCLC. Mutational status was known for 49% (191/392) of samples, including 41 patients with *EGFR* mutation and 62 patients with *KRAS* mutation.

To validate our findings from the initial cohort, we also performed similar studies using a separate patient TMA set ($n = 287$ patients). In this validation cohort, the mean age was 70 years and 76% (218/287) of patients underwent surgical resection for stage I–III NSCLC. As the information on race was not documented for most of cases in this cohort, race was not included in the analysis. The validation set differed from the discovery group in histology as it included very few cases of large-cell NSCLC, 1% (3/287). In addition, mutational status was known for 64% (185/287) of cases.

Immunohistochemical analysis of overall HHLA2 expression in lung cancer and normal lung tissues

To examine the expression profile of HHLA2 in primary lung tumors, we performed IHC and stained the TMA sets of NSCLC tumor tissues with the HHLA2-specific mAb clone 566.1. Some of the TMA sections did not have sufficient tumor tissue for accurate assessment and hence were not scored. Similar to a previous report (29), positive cases were defined as samples with positive membranous and cytoplasmic HHLA2 staining (intensity scores of 1, 2, or 3; Fig. 1B). In the discovery set, HHLA2 was found to be widely expressed in 62.8% ($n = 228/363$) NSCLC and HHLA2 was scored as 0 in 37.2% ($n = 135/363$), 1 in 31.7% ($n = 115/363$), 2 in 24.8% ($n = 90/363$), 3 in 6.3% ($n = 23/363$) of cases, respectively. Similarly, HHLA2 expression was positive in 71% ($n = 185/262$) of the cases in the validation cohort. Overall, HHLA2 was expressed in 66% of NSCLC cases when combining both cohorts.

We also reviewed 68 cases of normal lung tissues and did not detect HHLA2 expression in alveolar type 1 and type II cells, endothelial cells and smooth muscle in blood vessels. Occasional alveolar macrophages and club cells (also known as bronchiolar exocrine cells) were 1+ HHLA2 positive. Thus, in contrast to its wide expression in tumor specimens, HHLA2 protein was not expressed in most of normal lung tissues (Fig. 1C).

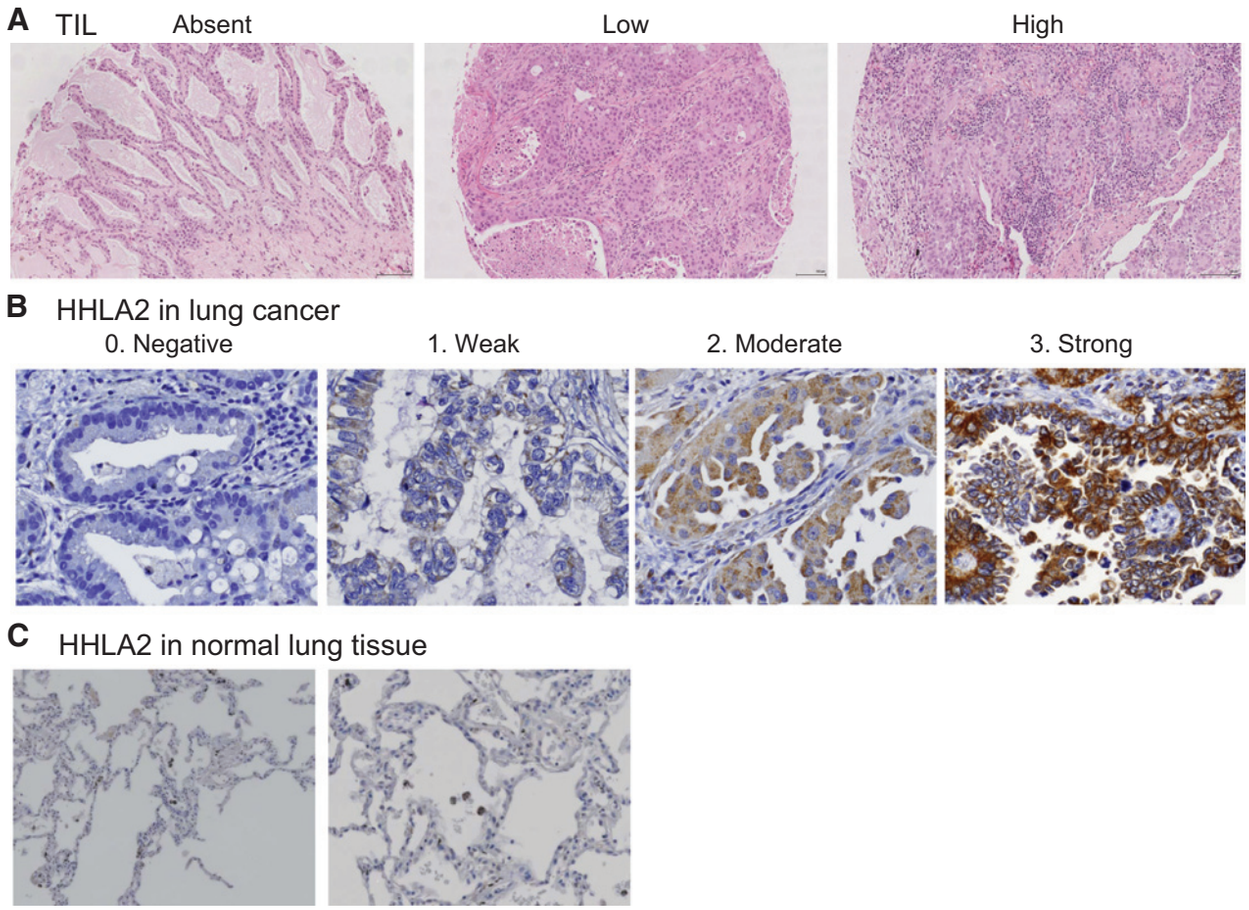

Figure 1.

A, Representative specimens showing TIL score of "0": absence of TILs, "Low": 1%–30%; and "High": >30%. A three-tiered scale based on the visual estimation of the proportion of lymphocytic infiltration in each histospot was used. **B,** Representative specimens demonstrating varying HHLA2 expression in lung cancer by IHC. The level of HHLA2 expression was graded as intensity of IHC staining: 0, absent staining; 1, weak staining; 2, moderate staining; 3, strong staining. **C,** Representative HHLA2 staining in normal lung tissues.

Table 1. Clinicopathologic characteristics of HHLA2 expression in patients with lung cancers from the discovery and validation cohorts

	Discovery cohort ($n = 392$)				Validation cohort ($n = 287$)		
Parameter	**HHLA2 Negative**	**HHLA2 Positive**	***P***	**Parameter**	**HHLA2 Negative**	**HHLA2 Positive**	***P***
Age, year	67.9	67.5	0.78	Age, year	70.5	70	0.47
Gender			0.60	Gender			0.09
Female ($n = 215$)	78 (36%)	137 (64%)		Female ($n = 180$)	46 (26%)	134 (74%)	
Male ($n = 141$)	55 (39%)	86 (61%)		Male ($n = 80$)	29 (36%)	51 (64%)	
Race			0.06	Race			
Asia ($n = 7$)	4 (57%)	3 (43%)		N/A			
AA* ($n = 18$)	8 (44%)	10 (56%)					
Hispanic ($n = 25$)	16 (64%)	9 (36%)					
White ($n = 230$)	87 (38%)	143 (62%)					
Histology			<0.0001	Histology			0.92
Adeno ($n = 290$)	91 (31%)	199 (69%)		Adeno ($n = 186$)	58 (31%)	128 (69%)	
Squam ($n = 31$)	20 (65%)	11 (35%)		Squam ($n = 29$)	8 (27%)	21 (73%)	
Large ($n = 18$)	16 (89%)	2 (11%)		Large ($n = 3$)	1 (33%)	2 (67%)	
Stage			0.09	Stage			0.39
I ($n = 252$)	85 (34%)	167 (66%)		I ($n = 157$)	43 (27%)	114 (73%)	
II ($n = 47$)	23 (49%)	24 (51%)		II ($n = 39$)	15 (38%)	24 (61%)	
III ($n = 35$)	10 (29%)	25 (71%)		III ($n = 22$)	7 (31%)	15 (69%)	
Lymph node (N per TNM staging)			0.35	Lymph node (N per TNM staging)			0.94
N0 ($n = 259$)	94 (32%)	165 (64%)		N0 ($n = 177$)	50 (28%)	127 (72%)	
N1 ($n = 35$)	14 (40%)	21 (60%)		N1 ($n = 27$)	7 (26%)	20 (74%)	
N2 ($n = 26$)	6 (23%)	20 (77%)		N2 ($n = 20$)	6 (30%)	14 (70%)	
Mutation status			0.04	Mutation status			0.01
EGFR ($n = 41$)	10 (24%)	31 (76%)		EGFR($n = 44$)	5 (11%)	39 (89%)	
KRAS ($n = 62$)	23 (37%)	39 (63%)		KRAS ($n = 66$)	24 (36%)	42 (64%)	
WT/WT ($n = 91$)	43 (47%)	48 (53%)		WT/WT ($n = 88$)	27 (31%)	61 (69%)	
TIL score			0.26	TIL score			0.03
Absent ($n = 13$)	3 (23%)	10 (77%)		Absent ($n = 24$)	6 (25%)	18 (75%)	
Low ($n = 238$)	94 (40%)	144 (60%)		Low ($n = 191$)	63 (33%)	128 (67%)	
High ($n = 108$)	35 (32%)	73 (68%)		High ($n = 44$)	6 (14%)	38 (86%)	

We then investigated the relationship between tumor HHLA2 expression and clinicopathologic variables in lung cancer (Table 1). There was no significant correlation between age and gender with tumoral HHLA2 expression.

HHLA2 expression by race

For the diverse racial groups in the discovery set, 36% (9/25) of Hispanic, 42.9% (3/7) of Asian, 55.6% (10/18) of African American, and 62.2% (143/230) of non-Hispanic white patients had HHLA2-positive lung cancers, respectively (Table 1). When pairwise comparisons were performed, the tumor expression of HHLA2 in non-Hispanic White patients was significantly higher than that in Hispanic patients ($P = 0.01$). Given lack of information in the validation cohort, similar analysis was not performed.

HHLA2 expression and histology

There are three major histologic subtypes for NSCLC: adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. We analyzed HHLA2 expression in different subtypes. In the discovery cohort, 68.6% (199/290) of adenocarcinoma, 35.4% (11/31) of squamous cell, and 11.1% (2/18) of large-cell NSCLC stained positive for tumor HHLA2, respectively (Table 1). The expression of HHLA2 in adenocarcinoma specimens was significantly higher than that in squamous cell ($P = 0.0002$) and large-cell NSCLC ($P < 0.0001$). In the validation cohort, the majority of adenocarcinoma (69%) was positive for HHLA2, which was similar to the initial cohort. However, there were very cases of large-cell NSCLC ($n = 3$) and HHLA2 expression was present in 73% of squamous cell cases. Thus, there was no difference among histology groups in this set (Table 1).

HHLA2 expression is associated with *EGFR* mutation status

EGFR and *KRAS* mutations are among the most common driver oncogenic alterations in NSCLC. Lung cancer patients with these mutations have distinct pathogenesis, clinical features, and disease course (3). We analyzed HHLA2 expression in different mutational categories. In the discovery cohort, positive HHLA2 staining was found in 75.6% (31/41) of *EGFR*-mutated, 62.9% (39/62) of *KRAS*-mutated, and 52.7% (48/91) of WT/WT (*EGFR* and *KRAS* wild-type) patients (Table 1). Among them, the HHLA2 expression in *EGFR*-mutated lung cancer was significantly higher than that in WT/WT patients ($P = 0.015$; Fig. 2). Similarly, in the validation cohort, *EGFR*-mutant tumors had significantly higher expression of HHLA2 when compared with WT/WT (89% vs. 69%, $P = 0.01$; Fig. 2).

HHLA2 expression and TIL density

The relationship between TILs and HHLA2 expression was examined. In the discovery set, HHLA2 was positive in 60% (144/238) of TIL^{low} cases and 68% (73/108) of TIL^{high} groups but the difference was not significant. However, a significantly higher TIL score correlated with higher HHLA2 positivity in the validation cohort (TIL^{low} vs. TIL^{high}, 67% vs. 86%, $P = 0.03$).

HHLA2 expression by stage and lymph node status

The expression profiles in different stages were also investigated. In the discovery set, 66.3% (167/252) of stage I, 51.1% (24/47) of stage II, and 71.4% (25/35) of stage III lung cancers were positive for HHLA2 expression and there was no significant difference among the three groups ($P = 0.09$). In addition, there was no significant difference in lymph node positivity with

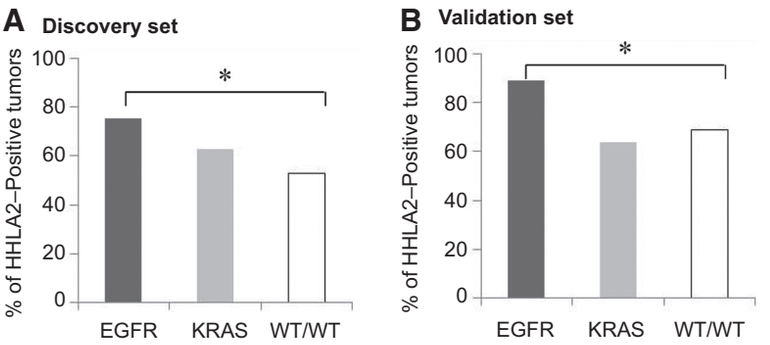

Figure 2.
Proportion of HHLA2-positive cases and pairwise comparison in different mutational groups in (**A**) Discovery cohort and (**B**) Validation cohort. *, $P < 0.05$.

positive HHLA2 expression in the subsets with different nodal status, NI, N2, and N3 ($P = 0.35$). Similarly, no relationship between HHLA2 expression and different stages or lymph node status was identified in the validation cohort.

Comparison of H-scores for different groups

Further analysis with Wilcoxon rank sum test was performed to test whether the median HHLA2 *H*-score was greater in one group than another.

In the discovery cohort (Table 2), the median HHLA2 H-scores for the *EGFR*-mutated lung cancers were significantly higher than that of WT/WT ($P = 0.015$), whereas no significant difference was found between *KRAS*-mutated and WT/WT groups ($P = 0.123$). Histology wise, adenocarcinoma specimens had significantly higher median H-scores than tumors with squamous ($P < 0.001$) or large-cell histology ($P < 0.001$). In addition, non-Hispanic White patients had significantly higher median HHLA2 tumor scores than Hispanic patients ($P = 0.034$). Moreover, the TIL^{high} group tended to have higher HHLA2 H-scores than the TIL^{low}, although not significantly in the whole discovery set. If only adenocarcinoma histology was considered, then HHLA2 scores were significantly higher in the TIL^{high} group ($P = 0.02$).

Similarly to the initial set, the median HHLA2 H-scores was significantly higher for the *EGFR*-mutated tumors than that of WT/WT ($P = 0.007$) in the validation cohort (Table 2). Furthermore, the TIL^{high} group had significantly higher HHLA2 H-scores than the TIL^{low} group in this set ($P = 0.02$; Table 2).

The tumor HHLA2 expression was also evaluated as mean H-scores and it was 81 for the discovery cohort and 60 for the validation set. The mean scores for each category are listed in Table 3. Taken together, our data suggest that HHLA2 is widely expressed in NSCLC and its tumoral expression is associated with *EGFR* mutation status in both cohorts.

Survival analysis in correlation with tumor HHLA2 expression and TIL scores in lung cancer

Survival data were available for 250 of the 393 patients in the discovery set. Neither tumor HHLA2 expression nor TIL scores was significantly associated with overall survival in NSCLC. In the EGFR-mutated subset, there was a trend toward higher HHLA2 expression with decreased overall survival ($P = 0.19$) but this was

Table 2. Wilcoxon rank sum test was performed to test whether the median HHLA2 H-score was greater in one group than the other

Discovery cohort			
Mutation	*EGFR*	WT/WT	*P*
Median H-score (interquartile range)	75 (20–200)	25 (0–100)	0.0151
Mutation	*KRAS*	WT/WT	*P*
Median H-score (interquartile range)	67.5 (0–150)	25 (0–100)	0.1231
Histology	Adeno	Squamous	*P*
Median H-score (interquartile range)	75 (0–200)	0 (0–30)	<0.0001
Histology	Adeno	Large cell	*P*
Median H-score (interquartile range)	75 (0–200)	0 (0–0)	<0.0001
Histology	Squamous	Large cell	*P*
Median H-score (interquartile range)	0 (0–30)	0 (0–0)	0.0802
Race	Non-Hispanic White	Hispanic	*P*
Median H-score (interquartile range)	60 (0–125)	0 (0–100)	0.0340
TIL	High	Low	*P*
Median H-score (interquartile range)	82 (0–200)	50 (0–125)	0.1060
Validation cohort			
Mutation	*EGFR*	WT/WT	*P*
Median H-score (interquartile range)	75 (27.5–137.5)	45 (0–100)	0.007
Mutation	*KRAS*	WT/WT	*P*
Median H-score (interquartile range)	25 (0–100)	45 (0–100)	0.389
Histology	Adeno	Squamous	*P*
Median H-score (interquartile range)	40 (0–100)	25 (0–50)	0.339
TIL	High	Low	*P*
Median H-score (interquartile range)	70 (22.5–100)	30 (0–100)	0.02

Table 3. Mean tumor HHLA2 H-scores for each category in lung cancers

Discovery cohort ($n = 392$)		Validation cohort ($n = 287$)	
Parameter	Mean HHLA2 H-score	Parameter	Mean HHLA2 H-score
Gender		Gender	
Female ($n = 215$)	81	Female ($n = 180$)	61
Male ($n = 141$)	81	Male ($n = 80$)	58
Histology		Histology	
Adeno ($n = 290$)	92	Adeno ($n = 186$)	60
Squamous ($n = 31$)	23	Squam ($n = 29$)	40
Large ($n = 18$)	15	Large ($n = 3$)	50
Stage		Stage	
I ($n = 252$)	85	I ($n = 157$)	66
II ($n = 47$)	77	II ($n = 39$)	43
III ($n = 35$)	82	III ($n = 22$)	51
TIL		TIL	
Absent ($n = 13$)	84	Absent ($n = 24$)	78
Low ($n = 238$)	77	Low ($n = 191$)	55
High ($n = 108$)	94	High ($n = 44$)	74
Mutation status		Mutation status	
EGFR ($n = 41$)	102	EGFR ($n = 44$)	89
KRAS ($n = 62$)	87	KRAS ($n = 66$)	52
WT/WT ($n = 91$)	65	WT/WT ($n = 88$)	56

not statistically significant. In addition, the logistic regression analysis revealed that the patients with *EGFR*/*KRAS* wild-type had significantly higher chance of death comparing to patients with *EGFR* mutation after adjusting for TIL scores [OR 3.402; 95% confidence interval (CI), 1.028–11.26; $P = 0.0153$].

EGFR and TIL status are independently associated with HHLA2 expression in lung adenocarcinoma

The multivariate analysis was performed in cases with lung adenocarcinoma because mutation tests, such as EGFR status, are not routinely performed in squamous cell lung cancer per standard of care. In the discovery set, both *EGFR* mutation status (WT/WT vs. *EGFR*; OR 0.32; 95% CI, 0.13–0.76; $P = 0.0079$) and TIL score (high vs. low; OR 2.441; 95% CI, 1.143–5.211; $P = 0.0021$) were independently associated with HHLA2 expression in lung adenocarcinoma ($n = 289$). Similarly, both *EGFR* mutation status (WT/WT vs. *EGFR*; OR 0.22; 95% CI, 0.13–0.22; $P = 0.015$) and TIL score (high vs. low; OR 3.22; 95% CI, 1.01–10.28; $P = 0.048$) were also independently associated with HHLA2 expression in the group of lung adenocarcinoma ($n = 186$) in the validation cohort.

Discussion

Analogous to other B7 molecules such as PD-L1, PD-L2, B7x, and B7-H3, HHLA2 belongs to the B7 family of coinhibitory molecules. These coinhibitory members of the B7 family of ligands interact with the CD28 family of receptors such as PD-1 and CTLA-4 with resultant T-cell inhibition. In our previous study (20), we examined HHLA2 expression on hematopoietic cells and found that HHLA2 protein was constitutively expressed on the surface of human monocytes and was induced on B cells after stimulation with lipopolysaccharide (LPS) and IFNγ, but not on dendritic cells or T cells. In this study, we described the highly frequent expression of HHLA2 in human lung cancer and its association with clinicopathologic characteristics and clinical outcome.

We revealed that HHLA2 was expressed widely in lung cancer, including its different subgroups. Lung cancer is considered as an immunogenic tumor and the expression of coinhibitory molecules represents one mechanism of tumor immune evasion. Unlike PD-L1, which is usually focally expressed in lung cancer, HHLA2 was homogeneously expressed in most (>90%) of the tumor cells in a given lung tumor sample and there was minimal variation in the staining intensity in a given sample. HHLA2 was expressed in 66% of lung cancers examined. In the discovery cohort, HHLA2 expression varied by ethnic background and there was a trend of higher expression in tumors from Caucasians than Hispanics. There could be at least two possible explanations: (i) evolutionary origin and (ii) carcinogenic exposure. Smoking is a carcinogen and PD-L1 is more highly expressed in smokers than in nonsmokers and whether this is a confounding factor in the higher expression of HHLA2 needs to be evaluated in a larger study. HHLA2 was also more highly expressed in adenocarcinoma than in the other subtypes of lung cancer in the discovery set although the trend was not significant in the validation cohort. This tendency of dissimilarity could be due to differences in the expression of genes involved in immune or inflammatory response between adenocarcinoma and squamous/large-cell cancers such as CFI, β-2 microglobulin, MHC class I and class II, serglycin, and MCP-3 or due to differences in neoantigen presentation between the subtypes resulting from a differential expression of MHC class I and class II genes including HLA-DRβ1, HLA-DRα, HLA-DPα1, HLA-E, and β2-microglobulin (31).

The sensitizing *EGFR* mutations in adenocarcinoma of the lung are associated with an excellent response to EGFR tyrosine kinase inhibitors (TKI). EGFR activation results in the upregulation of the immune checkpoint molecule, PD-L1, through the p-ERK1/2/p-c-Jun pathway (32, 33) and hence a similar mechanism could be postulated for HHLA2 overexpression in *EGFR*-mutated tumors. Indeed, we found that *EGFR* mutational status was significantly associated with higher HHLA2 expression in both discovery and validation cohorts. In addition, in the *EGFR*-mutated subgroup, patients with high HHLA2 tumors trended toward poorer survival, although this finding was not statistically significant. This could be important as HHLA2 expression might help define a subpopulation of patients who may do poorly despite TKIs for *EGFR*-mutated tumors.

TILs have been shown to be prognostic in some cancers but the predictive value of TILs remains controversial in lung cancer. In some studies, a higher density of TILs has been shown to have a lower disease-free and recurrence-free survival in early-stage lung cancer (34) but in other studies there was no correlation noted (30). Hence, we explored the correlation between TILs and HHLA2 expression. There was no association between the density of TILs and overall survival. However, in lung adenocarcinoma, there was a significant association between high TIL infiltration and HHLA2 expression after adjusting for mutational status. This suggests that there may be different factors involved in TILs recruitment and HHLA2 expression may at least partly be a response to TIL infiltration in lung adenocarcinoma.

HHLA2 binds to its putative receptors on a variety of immune cells, including T cells and antigen-presenting cells and subsequently inhibits proliferation and cytokine production of both human CD4 and CD8 T cells. TMIGD2 (29), also called immunoglobulin-containing and proline-rich receptor-1 (IGPR-1; ref. 35) or CD28 homology (CD28H; ref. 21), is one of receptors for HHLA2. We have recently proposed a model that tumor-expressed HHLA2 interacts not only with an unidentified receptor on activated T cells which leads to T-cell inhibition, but also with

TMIGD2 on endothelium which enhances tumor angiogenesis (24). The combination of T-cell–inhibitory function and wide expression in lung cancer suggests that HHLA2 could represent an important mechanism of tumor immune suppression and thereby HHLA2 might emerge as a relevant therapeutic target in human lung cancer.

Our study has limitations because it is a retrospective study and other etiologic factors such as smoking and treatment factors after relapse would impact overall survival. Because we used TMA sections in this study collected from predominantly early-stage, resected tumors with sufficient specimen available for banking, this may offer a somewhat limited tumor representation. Other factors affecting HHLA2 expression like T-cell subsets, expression of other coinhibitory molecules like PD-L1, B7-H3, and B7x were not measured which should be explored in further studies. Large studies are needed to better investigate the survival characteristics of HHLA2-overexpressing tumors. In summary, this is the first study that shows that the recently discovered immune checkpoint ligand, HHLA2, is highly expressed in lung cancer and *EGFR* mutation is associated with higher expression of HHLA2. Further studies are needed to explore the mechanisms of overexpression and targeting HHLA2 in lung cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Cheng, M. Janakiram, A. Borczuk, J. Lin, B. Halmos, R. Perez-Soler
Writing, review, and/or revision of the manuscript: H. Cheng, M. Janakiram, A. Borczuk, B. Halmos, R. Perez-Soler, X. Zang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Cheng, A. Borczuk, H. Liu
Study supervision: H. Cheng, R. Perez-Soler

Grant Support

The work was supported by the NIH Paul Calabresi Career Development Award for Clinical Oncology5K12CA132783-04 (to R. Perez-Soler and H. Cheng), the LUNGevity Foundation Targeted Therapeutics Award (to H. Cheng and B. Halmos), NIHR01CA175495 (to X. Zang), NIHR01DK100525 (to X. Zang), and Department of DefensePC131008 (to X. Zang).

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Received December 21, 2015; revised July 22, 2016; accepted July 26, 2016; published OnlineFirst August 23, 2016.

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. CA Cancer J Clin 2015;65:5–29.
2. Cheng H, Shcherba M, Kandavelou K, Liang Y, Liu H, Perez-Soler R. Emerging drugs for squamous cell lung cancer. Expert Opin Emerg Drugs 2015;20:149–60.
3. Inal C, Yilmaz E, Piperdi B, Perez-Soler R, Cheng H. Emerging treatment for advanced lung cancer with EGFR mutation. Expert Opin Emerg Drugs 2015;20:597–12.
4. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144:646–74.
5. Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. Science 2011;331:1565–70.
6. Zang X, Allison JP. The B7 family and cancer therapy: Costimulation and coinhibition. Clin Cancer Res 2007;13:5271–9.
7. Ohaegbulam KC, Assal A, Lazar-Molnar E, Yao Y, Zang X. Human cancer immunotherapy with antibodies to the PD-1 and PD-L1 pathway. Trends Mol Med 2015;21:24–33.
8. Zou W, Chen L. Inhibitory B7-family molecules in the tumour microenvironment. Nat Rev Immunol 2008;8:467–77.
9. Greenwald RJ, Freeman GJ, Sharpe AH. The B7 family revisited. Ann Rev Immunol 2005;23:515–48.
10. Chapoval AI, Ni J, Lau JS, Wilcox RA, Flies DB, Liu D, et al. B7-H3: a costimulatory molecule for T cell activation and IFN-gamma production. Nat Immunol 2001;2:269–74.
11. Zang X, Loke P, Kim J, Murphy K, Waitz R, Allison JP. B7x: a widely expressed B7 family member that inhibits T cell activation. Proc Natl Acad Sci U S A 2003;100:10388–92.
12. Sica GL, Choi IH, Zhu G, Tamada K, Wang SD, Tamura H, et al. B7-H4, a molecule of the B7 family, negatively regulates T cell immunity. Immunity 2003;18:849–61.
13. Prasad DV, Richards S, Mai XM, Dong C. B7S1, a novel B7 family member that negatively regulates T cell activation. Immunity 2003;18:863–73.
14. Zang X, Thompson RH, Al-Ahmadie HA, Serio AM, Reuter VE, Eastham JA, et al. B7-H3 and B7x are highly expressed in human prostate cancer and associated with disease spread and poor outcome. Proc Natl Acad Sci U S A 2007;104:19458–63.
15. Barach YS, Lee JS, Zang X. T cell coinhibition in prostate cancer: new immune evasion pathways and emerging therapeutics. Trends Mol Med 2011;17:47–55.
16. Hofmeyer KA, Ray A, Zang X. The contrasting role of B7-H3. Proc Natl Acad Sci U S A 2008;105:10277–8.
17. Jeon H, Ohaegbulam KC, Abadi YM, Zang X. B7x and myeloid-derived suppressor cells in the tumor microenvironment: a tale of two cities. Oncoimmunology 2013;2:e24744.
18. Janakiram M, Abadi YM, Sparano JA, Zang X. T cell coinhibition and immunotherapy in human breast cancer. Discov Med 2012;14:229–36.
19. Zang X, Sullivan PS, Soslow RA, Waitz R, Reuter VE, Wilton A, et al. Tumor associated endothelial expression of B7-H3 predicts survival in ovarian carcinomas. Mod Pathol 2010;23:1104–1112.
20. Zhao R, Chinai JM, Buhl S, Scandiuzzi L, Ray A, Jeon H, et al. HHLA2 is a member of the B7 family and inhibits human CD4 and CD8 T-cell function. Proc Natl Acad Sci U S A 2013;110:9879–84.
21. Zhu Y, Yao S, Iliopoulou BP, Han X, Augustine MM, Xu H, et al. B7-H5 costimulates human T cells via CD28H. Nat Commun 2013;4:2043.
22. Flajnik MF, Tlapakova T, Criscitiello MF, Krylov V, Ohta Y. Evolution of the B7 family: co-evolution of B7H6 and NKp30, identification of a new B7 family member, B7H7, and of B7's historical relationship with the MHC. Immunogenetics 2012;64:571–90.
23. Mager DL, Hunter DG, Schertzer M, Freeman JD. Endogenous retroviruses provide the primary polyadenylation signal for two new human genes (HHLA2 and HHLA3). Genomics 1999;59;255–63.
24. Janakiram M, Chinai JM, Zhao A, Sparano JA, Zang X. HHLA2 and TMIGD2: New immunotherapeutic targets of the B7 and CD28 families. Oncoimmunology 2015;4:e1026534.
25. Chinai JM, Janakiram M, Chen F, Chen W, Kaplan M, Zang X. New immunotherapies targeting the PD-1 pathway. Trends Pharmacol Sci 2015;36:587–95.
26. Borghaei H, Paz-Ares L, Horn L, Spigel DR, Steins M, Ready NE, et al. Nivolumab versus docetaxel in advanced nonsquamous non-small-cell lung cancer. N Engl J Med 2015;373:1627–39.
27. Garon EB, Rizvi NA, Hui R, Leighl N, Balmanoukian AS, Eder JP, et al. Pembrolizumab for the treatment of non-small-cell lung cancer. N Engl J Med 2015;372:2018–28.

28. Zhang Z, Kobayashi S, Borczuk AC, Leidner RS, Laframboise T, Levine AD, et al. Dual specificity phosphatase 6 (DUSP6) is an ETS-regulated negative feedback mediator of oncogenic ERK signaling in lung cancer cells. Carcinogenesis 2010;31:577–86.
29. Janakiram M, Chinai JM, Fineberg S, Fiser A, Montagna C, Medavarapu R, et al. Expression, clinical significance, and receptor identification of the newest B7 family member HHLA2 protein. Clin Cancer Res 2015; 21:2359–66.
30. Schalper KA, Brown J, Carvajal-Hausdorf D, McLaughlin J, Velcheti V, Syrigos KN, et al. Objective measurement and clinical significance of TILs in non-small cell lung cancer. J Natl Cancer Inst 2015;107: dju435.
31. McDoniels-Silvers AL, Nimri CF, Stoner GD, Lubet RA, You M. Differential gene expression in human lung adenocarcinomas and squamous cell carcinomas. Clin Cancer Res 2002;8:1127–38.
32. Chen N, Fang W, Zhan J, Hong S, Tang Y, Kang S, et al. Upregulation of PD-L1 by EGFR activation mediates the immune escape in EGFR-driven NSCLC: implication for optional immune targeted therapy for NSCLC patients with EGFR mutation. J Thorac Oncol 2015;10:910–23.
33. Tang Y, Fang W, Zhang Y, Hong S, Kang S, Yan Y, et al. The association between PD-L1 and EGFR status and the prognostic value of PD-L1 in advanced non-small cell lung cancer patients treated with EGFR-TKIs. Oncotarget 2015;6:14209–19.
34. Horne ZD, Jack R, Gray ZT, Siegfried JM, Wilson DO, Yousem SA, et al. Increased levels of tumor-infiltrating lymphocytes are associated with improved recurrence-free survival in stage 1A non-small-cell lung cancer. J Surg Res 2011;171:1–5.
35. Rahimi N, Rezazadeh K, Mahoney JE, Hartsough E, Meyer RD. Identification of IGPR-1 as a novel adhesion molecule involved in angiogenesis. Mol Biol Cell 2012;23:1646–56.