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Expression, clinical significance, and receptor identification of the newest B7 family member HHLA2 protein

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Abstract

Purpose—HHLA2 (B7H7/B7-H5/B7y) is a newly identified B7 family member that regulates human T cell functions. However, its protein expression in human organs and significance in human diseases are unknown. The objective of this study was to analyze HHLA2 protein expression in normal human tissues and cancers, its prognostic significance, to explore mechanisms regulating HHLA2 expression, and to identify candidate HHLA2 receptors.

Experimental Design—An immunohistochemistry protocol and a flow cytometry assay with newly generated monoclonal antibodies were developed to examine HHLA2 protein. HHLA2 gene copy number variation was analyzed from cancer genomic data. The combination of

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Disclosure of Potential Conflicts of Interest

The authors declare no competing financial interests.

Author Contributions

M.J and J.M.C. performed experiments, analyzed data, and wrote the manuscript. S.F., R.M., and E.C. interpreted the slides. A.F., C.M. and S.C.A conducted bioinformatic analysis. H.J., K.C.M., R.Z. and A.Z. provided new reagents. J.A.S. and X.Z. supervised the study and wrote the manuscript.

bioinformatics analysis and immunological approaches was established to explore HHLA2 receptors.

Results—HHLA2 protein was detected in trophoblastic cells of the placenta and the epithelium of gut, kidney, gallbladder and breast, but not in most other organs. In contrast, HHLA2 protein was widely expressed in human cancers from the breast, lung, thyroid, melanoma, pancreas, ovary, liver, bladder, colon, prostate, kidney, and esophagus. In a cohort of 50 patients with stage I–III triple negative breast cancer, 56% of patients had aberrant expression of HHLA2 on their tumors, and high HHLA2 expression was significantly associated with regional lymph node metastasis and stage. The Cancer Genome Atlas revealed that HHLA2 copy number gains were present in 29% of basal breast cancers, providing a potential mechanism for increased HHLA2 protein expression in breast cancer. Finally, Transmembrane and Immunoglobulin Domain Containing 2 (TMIGD2) was identified as one of the receptors for HHLA2.

Conclusion—Wide expression of HHLA2 in human malignancies, association with poor prognostic factors and its T cell coinhibitory capability, suggests that the HHLA2 pathway represents a novel immunosuppressive mechanism within the tumor microenvironment and an attractive target for human cancer therapy.

Introduction

The past decade has witnessed an important change in the understanding of T cell biology and tumor immunology with the recognition of immune checkpoints through the B7-CD28 pathways (1–3). B7-1/B7-2/CD28/CTLA-4 is the prototypic B7/CD28 pathway. This loop of costimulation and coinhibition is critical for regulating the early stages of T cell responses in lymphoid organs. Several additional B7 family members are believed to play important roles in peripheral tolerance and tumor immune evasion– PD-L1 [B7-H1 (4,5)], B7-H3 (6) and B7x [B7-H4/B7S1 (7–9)]. The ligands PD-L1, B7-H3 and B7x function by inhibiting effector T cells in the peripheral tissues (10,11). These ligands are expressed in various human cancers and their expression can lead to immune tolerance in the tumor microenvironment by inhibiting T cell proliferation and function (1,12–14). In addition, B7x can interact with myeloid-derived suppressor cells (15,16), which may also promote tumor growth. Clinically, higher expression of these ligands is associated with a poor prognosis in various malignancies. Based on these functional and clinical observations, blocking some of the B7-CD28 pathways has yielded some therapeutic success in human malignancies. The anti-CTLA-4 antibody achieved clinical responses in melanoma (17) while anti-PD-L1 or anti-PD-1 antibodies have shown responses in melanoma, renal cell cancer, and non-small cell lung cancer (18–20). The therapeutic responses seen in these patients are durable and they are longer than chemotherapy or other targeted agents.

HERV–H LTR-associating 2 (HHLA2, also called B7H7/B7-H5/B7y)has been recently discovered as the newest member of the B7 family (21–23) and has 23–33% similarity in amino acid sequence with the other B7 molecules (21). This ligand is the only B7 family member that is found in humans but not in mice. It is constitutively expressed on the surface of human monocytes and is induced on B cells. HHLA2 binds to its putative receptor(s) on a variety of immune cells including CD4 and CD8 T cells and antigen-presenting cells (21). Similarly to B7-H3, both a T cell coinhibitory role as well as a costimulatory role has been

reported for this ligand (21,22). There have been no reports published on the protein expression of HHLA2 in normal human tissues or cancers and the clinical significance of this ligand is not currently understood. Here we present the first study on the expression of this protein in peripheral tissues as well as on numerous human cancers. Further, we have demonstrated that the overexpression of this protein in tumors is associated with worse clinical outcomes in breast cancer patients. One of the mechanisms whereby HHLA2 is overexpressed in human cancer seems to be gene copy number variation. Finally, we discovered Transmembrane and Immunoglobulin Domain Containing 2 (TMIGD2) is one of the receptors for HHLA2.

Methods

Patients and Samples

Normal and cancerous tissue microarrays were purchased from Imgenex Corp for the analysis of HHLA2 expression in human tissues. For the TNBC cohort 50 cases were selected from our tumor registry between 2002 -2011 who were diagnosed with local or locally advanced breast cancer diagnosed at our institution between 2002–2011. All these patients underwent surgery as the primary treatment followed by chemotherapy or radiotherapy or both. Tissue blocks from the mastectomy or lumpectomy specimen were located and slides were made from the same section. This was done so as to enable us to determine whether HHLA2 expression in the primary tissue is associated with prognostic features. Using retrospective chart review, the relevant clinical data were collected from the files. Hormone receptor status and Her2 status were also obtained by retrospective review. All protocols were reviewed and approved by the Institutional Review Board.

Monoclonal Antibodies and Immunohistochemistry

A mouse monoclonal antibody against HHLA2 (Clone 566.1, IgG1) and 3T3 cell lines expressing HHLA2 or CTLA-4 were recently generated (21). Cell blocks of the 3T3 cells expressing either HHLA2 or CTLA-4 were prepared for IHC controls by fixing the cells with 4% paraformaldehyde and then embedding them in HistoGel (Thermo Scientific). These samples were then embedded in paraffin and cut onto slides and used as positive and negative controls, respectively. These controls were then stained for HHLA2 using our mAb. Briefly, 4–5 μm thick formalin fixed paraffin embedded specimens slides were used. The tissue sections were deparaffinized in xylene and dehydrated through graded alcohols to water. The samples were boiled in citrate buffer (pH 6.0) using a microwave for 2 minutes and then incubated in an antigen retrieval steam chamber between 80–100°C for 30 minutes. To block endogenous peroxidase activity all of the sections were treated with 3% hydrogen peroxide for 10 min. Nonspecific binding of IgG was blocked by using protein serum block solution (Dako). The sections were incubated with anti-HHLA2 mAbs for 30 minutes. Then they were incubated with Dako envision+ HRP-labeled anti-mouse polymer. Signals were generated by incubation with 3, 3′-diaminobenzidine. Finally the sections were counterstained with hematoxylin and observed under the microscope. The slides were reviewed in tandem by a breast pathologist and by an oncology physician trained in breast pathology.

Cell Lines and FACS

Cell lines were cultured in either complete RPMI or DMEM. Cells were stained with anti-HHLA2 mAb 566.1 and then with a secondary anti-mouse IgG-APC conjugated antibody. For receptor binding, 3T3 cells expressing HHLA2-YFP or CTLA-4-YFP were incubated with TMIGD2-Ig or control Ig for 45 min on ice and then stained with anti-human IgG-APC. Similarly, 3T3 cells expressing TMIGD2-YFP or CTLA-4-YFP were incubated with HHLA2-Ig or control Ig for 45 min on ice and then stained with anti-human IgG-APC. Samples were analyzed by a BD FACSCalibur flow cytometer and FlowJo software.

Bioinformatic Analysis

The NCBI database was queried for proteins of the immunoglobulin family with homologues in humans and monkeys, but not in mice or rats. The sequences of the resulting list of proteins were analyzed by various domain-prediction programs to determine if these proteins contained Ig, IgC, IgC-like, IgV, or IgV-like domains. The list was further refined by excluding proteins that did not contain a transmembrane domain. MacVector 10.6. was used for sequence alignment and homology comparison. The phylogenetic tree was generated by PAUP(4.0b10) after removal of significant inserts and trimming C- and N-terminal extensions from sequence alignments(24). Motifs and domains were analyzed with EMBL-EBI tools, SMART, and CBS Prediction. For gene copy number variations, the cBioPortal for Cancer Genomics database and the Cancer Genome Atlas were analyzed.

Fusion Protein Production and Purification

TMIGD2-Ig fusion protein was prepared by PCR-amplifying the extracellular domain of the protein without the signal peptide. The amplified product was inserted into a human IgG1 Fc tag of plasmid pMT/BiP as described previously (7). *Drosophila* Schneider 2 cells were co-transfected with this construct and a plasmid inducing hygromycin resistance. The fusion protein was expressed in Express Five serum-free medium (Life Technologies) and purified using Protein G Plus Agarose columns (Pierce). The purity of the fusion protein was confirmed by SDS-PAGE.

Statistical Analysis

For continuous variables t-test was used when the normality assumption was met and the rank sum test and median was calculated for those, which violated normality. Chi-squared test was used to analyze the association between categorical variables and if the expected frequency is less than 5 in more than 20% of the cells, Fisher's exact test was used. All p values <0.05 was considered statistically significant.

Results

Normal human tissue expression of HHLA2

The expression of HHLA2 protein in human tissues is unknown at present. To examine the protein expression, we used our recently-generated HHLA2-specific monoclonal antibody clone 566.1 to develop an immunohistochemistry protocol to detect HHLA2 in formalin fixed paraffin-embedded specimens in which 3T3 cells expressing HHLA2 or CTLA-4

served as positive and negative controls, respectively (Figure S1). We used this technique to stain tissue microarrays of normal human organs (Table 1). Our results demonstrated that the majority of normal organs did not express HHLA2 at the protein level; however, trophoblastic cells of the placenta and epithelial cells of the gut, kidney, gallbladder and breast expressed this ligand (Figure 1 and Table 1). While primary and secondary lymphoid organs were largely negative, a few scattered cells appeared to stain positively in these samples. These results reveal that endogenous HHLA2 protein is absent in most normal tissues, but mainly expressed on epithelial cells of a few tissues.

HHLA2 is widely expressed in various human cancers

HHLA2 is able to inhibit both human CD4 and CD8 T cell functions(21),therefore it is possible that human cancer may exploit this pathway as an immune evasion mechanism. As no knowledge currently exists about HHLA2 protein in human cancer tissues, we next examined the expression of HHLA2 in human cancer tissues using immunohistochemical staining of tissue microarrays of common cancers from various organs (Figure 2). HHLA2 was expressed in 50% or more of cancers from the breast (7 of 10), lung (6 of 9), thyroid (6 of 9), melanoma (5 of 9), ovary (4 of 8), and pancreas (5 of 10) samples (Table 2). The localization of the protein was membranous and cytoplasmic in tumor cells (Figure 2). HHLA2 protein was also expressed in cancers of the liver, bladder, colon, prostate, kidney, and esophagus (Table 2 and Figure 2). In addition, we found that of the 20 human cell lines examined in breast cancer and hematological malignancies of leukemia and lymphoma, 12 expressed HHLA2 on their surface by flow cytometry (Table S1). These results demonstrate that HHLA2 protein is widely over-expressed in human cancers and has a high prevalence in certain malignancies.

HHLA2 expression in triple negative breast cancer

We also evaluated HHLA2 expression in a cohort of 50 patients with early stage triple negative breast cancer (TNBC) because of the limited therapeutic options for this breast cancer subtype. The characteristic of the patient population is shown in Table 3. The mean age of the patient population was 57.6 years, average tumor size was 2.35 cm (SD: 1.49), 70% had at least 1 positive lymph node (n =35), and the distribution of American Joint Committee on Cancer (AJCC) Stage was stage 1 in 20%, stage 2 in 50%, and stage 3 in 30%. All patients underwent primary therapy (i.e. mastectomy or lumpectomy and radiotherapy), and most of them 92%received adjuvant chemotherapy.

Using immunohistochemical staining we found that within a given TNBC tumor, the expression and intensity of HHLA2 protein was quite homogeneous throughout the tumor. Hence HHLA2 expression was graded based on intensity of staining between 0 and 3. Grades of 0 and 1 had no or minimal staining, respectively, whereas 2 and 3 had moderate and strong membranous/cytoplasmic staining. 0 and 1 were considered to be negative or low expression while 2 and 3 were considered to be high expression (Figure 3).

In the 50 TNBC samples stained, HHLA2 was graded as 0 in 12% (n= 6), 1 in 32% (n= 16), 2 in 40% (n=20) and 3 in 16% (n=8) of the tissue sections (Table 3). When classified as a binary variable, 56% (n=28) exhibited high (grades 2 or 3) and 44% (n=22) had low

expression (grades 0 or 1) of HHLA2. In the bivariate analysis, high HHLA2 expression was associated with lymph node positivity (0 vs. ≥1, p=0.04) and advanced stage of the disease (p = 0.03) at the time of diagnosis, features known to be associated with an increased risk of recurrence. HHLA2 expression was not related to age or to the size of the tumor. Together, these data demonstrate that more than half of TNBC tumors have HHLA2 overexpression and that patients with higher levels of HHLA2 on their tumors are significantly more likely to have the disease spread and at an advanced stage.

HHLA2 gene copy number variations in triple negative breast cancer

HHLA2 was overexpressed at the protein level in breast cancer, but the mechanism(s) up-regulating the expression in cancer cells is unknown. Therefore we sought to determine whether gene amplification was a potential mechanism of overexpression. By analyzing the cBioPortal for Cancer Genomics database (25,26), we found that HHLA2 gene alterations were present in 18.8% and 23% of all cases with breast cancer in The Cancer Genome Atlas (TCGA) (27) and in the TCGA provisional database studies, respectively. Since TNBC is predominantly comprised of the basal subtype, we compared the copy number gain of the HHLA2 locus in basal vs. non basal breast cancer using the TCGA registry. HHLA2 was altered in 32% of the basal subtype which is almost twice the frequency observed in all breast cancers independent of their subtype (18%). The vast majority of HHLA2 copy number variations (CNVs) in basal breast cancers were amplifications or gains (>95%). Hence, given that HHLA2 protein up-regulation is present in approximately 56% of our samples, our results suggest that one mechanism of up regulation of HHLA2 protein in TNBCs is gene copy number gain.

Identification of immunoglobulin domain–containing protein 2 (TMIGD2) as one of the receptors for HHLA2

Receptors for HHLA2 are widely expressed on both naïve and activated T cells as well as dendritic cells, monocytes, and B cells (21). As HHLA2 is a member of the immunoglobulin superfamily and has orthologs in humans and monkeys but not in mice or rats, we hypothesized the receptors for HHLA2 may belong to the immunoglobulin superfamily and have the same phylogenetic pattern due to co-evolution.

From more than 500 members of the immunoglobulin superfamily (28,29), a list was compiled of the immunoglobulin family members expressed in humans and monkeys but not in mice or rats. This list was further refined by only including members with predicted transmembrane domains and we then stably transfected these candidates into 3T3 cells. We tested their ability to bind to HHLA2-Ig fusion protein. Using flow cytometry, we found that HHLA2-Ig bound to cells expressing Transmembrane and Immunoglobulin Domain Containing 2 (TMIGD2, Figure 4A–B). A TMIGD2-Ig fusion protein was then constructed in which the extracellular domain of TMIGD2 linked to the Fc region of human IgG1 (TMIGD2-Ig). TMIGD2-Igbound strongly to 3T3 cells expressing HHLA2 but not CTLA-4 in flow cytometer (Figure 4B). TMIGD2 contains an N-terminal signal peptide, an extracellular IgV-like domain, three potential sites for N-linked glycosylation, a transmembrane region, and a cytoplasmic tail with four potential sites for phosphorylation and a possible site for SH3 domain recognition. (Figure 4A). By sequence analysis, we

found TMIGD2, the immunoglobulin-containing and proline-rich receptor-1 (IGPR-1)(30), and CD28 homologue (CD28H)(22) are the same molecule. IGPR-1 was originally identified as a adhesion molecule involved in angiogenesis (30), while CD28H was recently reported as a receptor by a high-throughput screen of transmembrane proteins (22). Thus TMIGD2/IGPR-1/CD28H is one of the receptors for HHLA2.

Discussion

The B7 ligand family and the CD28 receptor family are the major driving force of T cell costimulation and coinhibition. These molecules have been intensely studied for their potential clinical impact in human malignancies, especially with regards to ectopic tumor cell expression of negative coinhibitory molecules. Here we present the first study on the protein expression, clinical significance, and mechanism of up-regulation of HHLA2 in human tissues and cancers. The results reveal that HHLA2 is a suitable target for cancer therapy.

HHLA2 appears to have limited expression in normal tissues. Most human tissues we tested were negative for HHLA2 protein. A few organs, including intestines, breast, kidney, gallbladder and placenta, express HHLA2, particularly on epithelial cells. We previously showed that blood B cells activated by LPS and IFN expressed HHLA2 (21). IHC staining of tissue microarrays of normal human organs showed there were only scattered HHLA2 positive cells in primary and secondary lymphoid organs. Currently it is unknown whether activated B cells in blood and in tissues have different expression pattern of HHLA2. Tissue-expressed PD-L1, another B7 family member, was recently shown to protect against gut inflammation in mouse models (31). Future investigation is warranted to determine whether HHLA2 in the intestines contributes to the immune tolerance or intestinal inflammation. We observed high expression of HHLA2 in the placental tissue, suggesting it may play a role in fetal maternal immune tolerance. Interestingly, HHLA2 polymorphisms are associated with Autism spectrum disorders, a disease whose etiology remains poorly understood (32). Immune dysregulation was recently proposed to contribute to the rapid development of Autism spectrum disorder in genetically susceptible children (32). Further study will also be required to determine whether HHLA2 is involved in the development of Autism spectrum disorder.

HHLA2 was highly expressed in most malignant tissues. This wide expression in various tumors indicates that HHLA2 expression may be a critical step in tumor evolution and that HHLA2 could confer a survival advantage to tumors via suppression of host anti-tumor immunity. Since breast cancer had a high expression of HHLA2, we evaluated its clinical impact in our TNBC cohort. Analysis of HHLA2 expression in the breast cancer cohort revealed that HHLA2 was highly expressed in 56% of triple negative breast cancer patients; about 80% of TNBC are basal subtype by gene expression. Tumors with high HHLA2 expression exhibited uniform expression of HHLA2 in the membrane or cytoplasm with minimal intratumoral heterogeneity. In contrast, other B7 ligands such as PD-L1 show focal expression and significant tumor heterogeneity. This uniform expression of HHLA2 in tumors suggests it may be a primary change in tumors during tumor evolution and/or its expression could be induced by factors in the tumor microenvironment. There are at least

two possible mechanisms governing the up-regulation of HHLA2 expression: inflammatory stimulation and gene copy number gain. HHLA2 expression is induced on B cells and enhanced on monocytes by stimulation with LPS and IFN-γ (21). Our gene copy number analysis revealed that the vast majority of HHLA2 copy number variations in basal breast cancer were gains (>95%), suggests that gene copy number gain is one of the mechanisms up-regulating HHLA2 expression in cancer. Expression of HHLA2 was associated with two prognostic factors – advanced stage and lymph node positivity, but it was not related to the size of the tumor by bivariate analysis. The association of HHLA2 with lymph node positive disease suggests that this may be a change that is required for early tumor invasion.

We have previously reported that receptors for HHLA2 exist on a wide variety of immune cells including T cells, B cells, monocytes, and DCs. We utilized bioinformatics analysis and immunology approaches to determine that TMIGD2/IGPR-1/CD28H is one of the receptors for HHLA2. This strategy was used because of the unique phylogenetic profile of HHLA2 and the observation that ligand and receptor pairs tend to co-evolve. It is unlikely, however, that tumor-expressed HHLA2 inhibits the immune system through the interaction with TMIGD2/IGPR-1/CD28H, as TMIGD2 is reported to be expressed on naïve T cells but not on other immune cells and is lost rapidly after activation of naïve T cells (22), whereas tumor-infiltrating T cells are not naïve cells. Further study will be required to discover coinhibitory receptors on immune cells that tumor cell-expressed HHLA2 interacts with to inhibit anti-cancer immunity.

Since this is a cross sectional study and this is a small cohort of TNBC patients analyzed and hence limited inference could be drawn from this cross sectional study. The association of HHLA2 positivity and advanced stage could have been driven by the association of HHLA2 with lymph node positivity. Hence larger studies are needed to confirm these associations and other important characteristics like disease free and overall survival in triple negative and other breast cancers.

In summary, HHLA2 has restricted tissue expression, but is over-expressed in various human cancers. Importantly, its expression is associated with important prognostic factors in breast cancer. Furthermore, cancer cells may amplify the HHLA2 gene in order to increase its expression and to undermine host immunity. Finally, our results suggest that HHLA2 in human cancers is a suitable target for cancer immunotherapies such as checkpoint blockade and antibody-drug conjugate treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational relevance

The B7 and CD28 families are critical in regulating T cell responses and antibodies against PD-1, PD-L1 and CTLA-4 have successfully led to durable clinical remissions in various malignancies. HHLA2 was recently discovered as a new member of the B7 family and shown to regulate T cell function. Here we showed that HHLA2 had limited expression in normal human tissues but was expressed in various human cancers. We demonstrated its expression on tumor cells was associated with increased lymph node metastases in limited stage triple negative breast cancer (TNBC). Analyzing the TCGA database revealed TNBC had higher HHLA2 copy number gains than other subtypes of breast cancer, providing a mechanism of overexpression. Finally, we identified one of the receptors for HHLA2.This is the first study to report HHLA2 expression in human cancers and clinical significance, and together with its previously reported T cell co-inhibition properties show that this could contribute to tumor immune suppression and be a target for cancer immunotherapy.

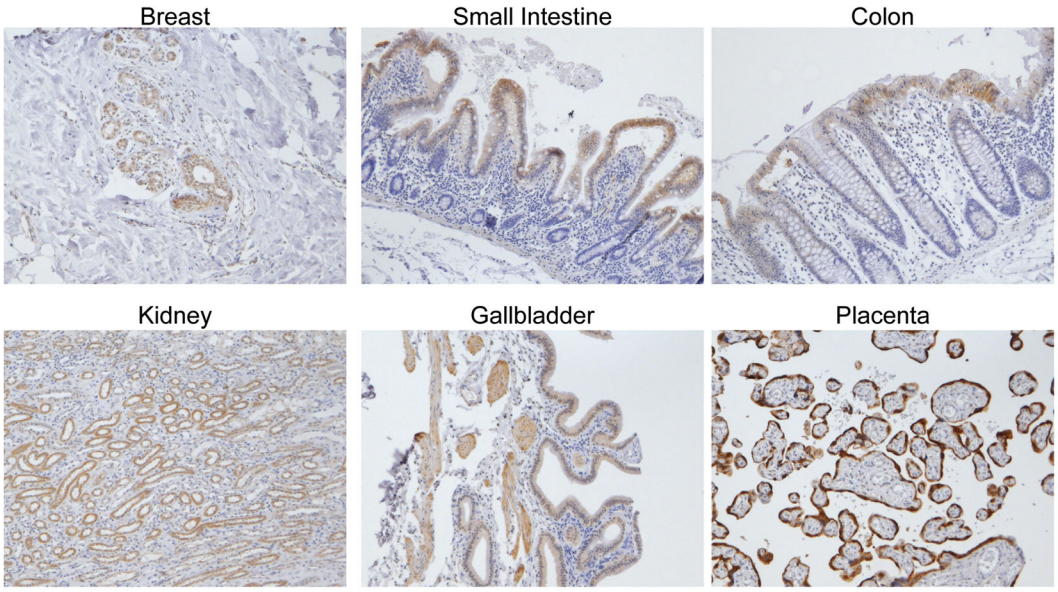

Figure 1. Normal human tissues that stained positively for expression of HHLA2 protein

Images were acquired at 10x. A summary of staining results from tissue microarrays is shown in Table 1.

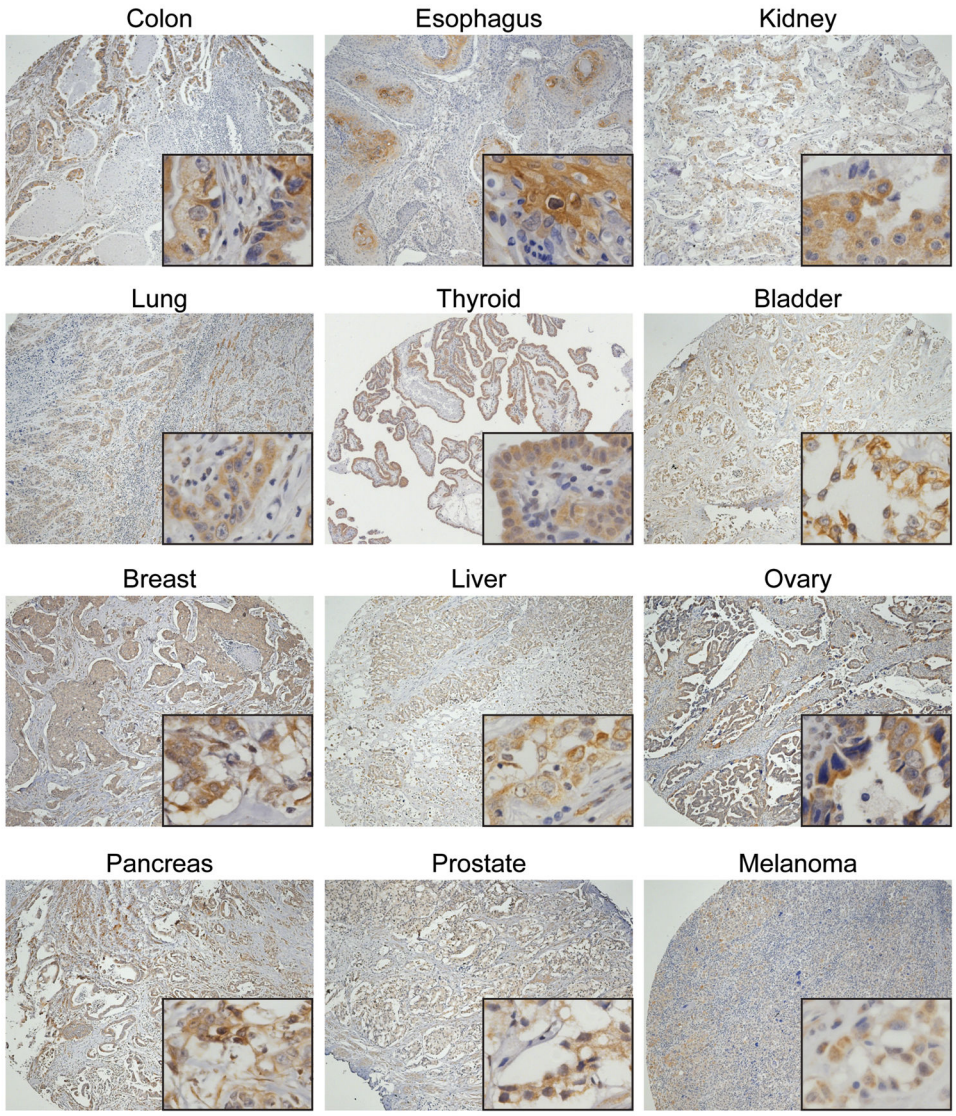

Figure 2. Examples of human cancers that stained positively for HHLA2 protein expression

Full size images were acquired at 5x and zoom-in images were originally acquired at 40x. A summary of the tissue microarray staining data is shown in Table 2.

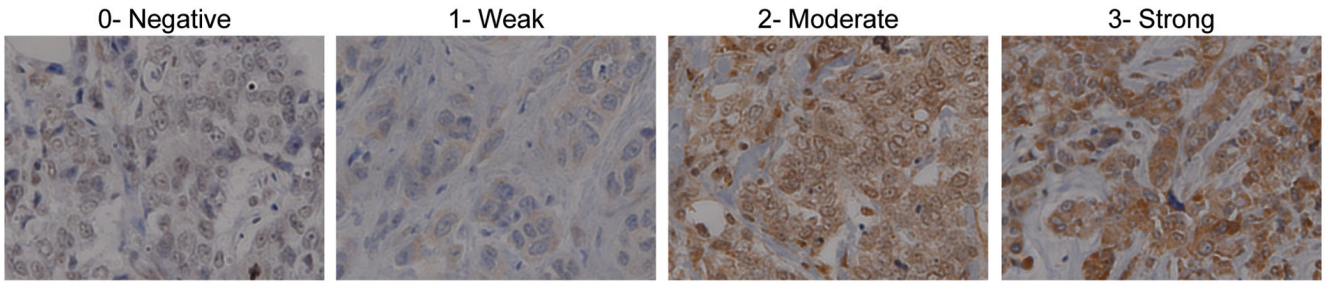

Figure 3. HHLA2 expression in human triple negative breast cancer (TNBC)

Tumors from a cohort of patients with triple negative breast cancer were stained for HHLA2 protein expression. The level of HHLA2 protein was graded as follows: 0 – absent staining, 1 – weak to minimal staining, 2 – moderate staining, 3 – strong staining.

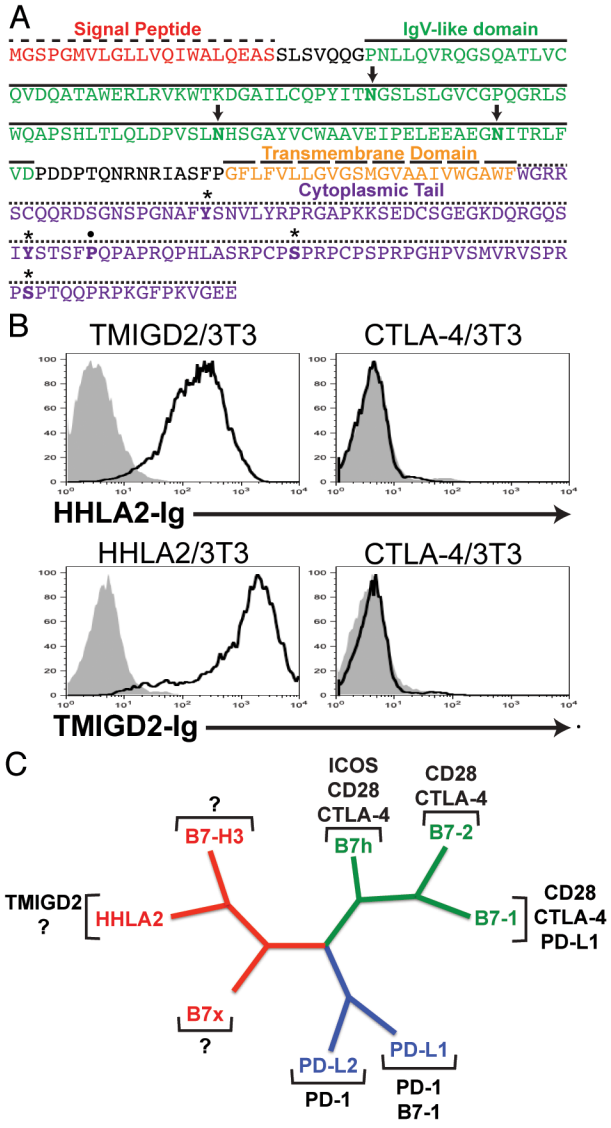

Figure 4. TMIGD2 is an immunoglobulin family member that binds to HHLA2

A) TMIGD2 is composed of a signal peptide, a single extracellular immunoglobulin domain, transmembrane domain, and a cytoplasmic tail. There are 3 predicted sites of N-linked glycosylation within the Ig domain (arrows) and four sites of predicted phosphorylation and one potential SH3-binding domain within the cytoplasmic tail. B) TMIGD2-Ig binds to HHLA2-expressing 3T3 cells but not to CTLA-4-expressing 3T3 cells. Cells were stained with HHLA2-Ig (open histograms) or control Ig (shaded histograms) followed by anti-human IgG-APC. C).Phylogenetic tree of the human B7 and CD28 families. The phylogenetic comparison of human B7 molecules was generated by PAUP and was divided into three groups. Receptors for human B7 molecules are also shown.

Table 1

HHLA2 protein expression in normal human organs assessed by immunohistochemistry on tissue microarrays.

Normal Tissues (Number Positive/Total Cores Analyzed)		
Placenta (3/3) (Trophoblastic cells)	Colon (3/3) (Epithelial cells)	Breast (3/3) (Ductal epithelium)
Small Intestine (2/2) (Epithelial cells)	Kidney (4/5) (Tubular epithelium)	Gallbladder (5/11) (Epithelial cells)
Adrenal Gland (0/2)	Aorta (0/2)	Brain (0/7)
Esophagus (0/2)	Larynx (0/9)	Liver (0/3)
Lung (0/3)	Lymph Node (0/12)	Ovary (0/1)
Pancreas (0/2)	Prostate (0/3)	Skin (0/3)
Spleen (0/3)	Stomach (0/3)	Subcutis (0/2)
Thymus (0/2)	Thyroid (0/2)	Tonsils (0/2)
Umbilical Cord (0/2)	Uterine Cervix (0/4)	Uterus (0/9)

Table 2

HHLA2 protein expression in human cancers assessed by immunohistochemistry on tissue microarrays.

Cancer Samples (Number Positive/Total Cores)		
Breast (7/10)	Lung (6/9)	Thyroid (6/9)
Malignant Melanoma (5/9)	Pancreas (5/10)	Ovary (4/8)
Liver (4/10)	Bladder (4/10)	Colon/Rectum (3/8)
Prostate (3/9)	Kidney (2/6)	Esophagus (2/10)
Endometrial (0/9)	Gallbladder (0/10)	Larynx (0/10)
B cell Lymphoma (0/10)	Stomach (0/10)	Uterine Cervix (0/10)

Table 3

Clinicopathologic features of HHLA2 expression in human triple negative breast cancer (TNBC) cohort.

Demographics	**Entire Population**	**Population by HHLA2 Expression**		***p* value**
		High (n = 28)	**Low (n = 22)**	
Mean Age (95% CI)	57.6	57.1 (52.1–62.1)	58.4 (53–63.8)	0.71
Mean Size (25th–75th percentile)	2.4	2.6 (1.8–4)	2.15 (1.4–2.5)	0.12
Lymph nodes, n (%)				0.04
0	15 (30%)	5 (18%)	10 (46%)	
≥ 1	35 (70%)	23 (82%)	12 (54%)	
AJCC Stage				0.03
1	10 (20%)	2 (7%)	8 (36%)	
2	25 (50%)	15 (54%)	10 (46%)	
3	15 (30%)	11 (39%)	4 (18%)	