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**Comprehensive molecular profiling of the B7 family of immune-regulatory
ligands in breast cancer**

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Keywords

B7 family, breast cancer, check point, CD28 family, cancer immunotherapy

Abstract

The B7 gene family has crucial roles in the regulation of adaptive cellular immunity. In cancer, deregulation of co-inhibitory B7 molecules is associated with reduced anti-tumor immunity and cancer immune evasion. FDA approval of cancer immunotherapy antibodies against cytotoxic T lymphocyte-associated protein 4 (CTLA-4) and programmed cell death-1 (PD-1)—both ligands of the B7 family—demonstrate the impact of these checkpoint regulators in cancer. Using data from cBioPortal, we performed comprehensive molecular profiling of the ten currently known B7 family proteins in 105 different cancers. B7 family members were

amplified in breast cancer: with B7 mRNA levels up-regulated in a cohort of 1098 patients with all types of breast cancer and in 82 patients with triple-negative breast cancer. Promoter methylation analysis indicated an epigenetic basis for deregulation of certain B7 family genes in breast cancer. Moreover, patients with B7-H6 genomic alterations had significantly worse overall survival, and certain clinical attributes were associated with B7-H6 expression, which indicates that B7-H6 may be a potential target for breast cancer immunotherapy. Finally, using network analysis (based on data from cBioportal), we identified BTLA, MARCH8, PLSCR1 and SMAD3 as potentially involved in T cell signaling under regulation of B7 family proteins.

Introduction

An effective immune response involves activation of both innate and adaptive immunity (which requires an array of immunocytes) followed by dampening of the immune response to avoid unnecessary damage to the host. T cells are essential to adaptive immunity, and are activated by two classical signals: antigen recognition (signal one), where peptides presented by the major histocompatibility complex (MHC) are recognized by T cell receptors (TCRs); and co-stimulation (signal two), which involves the combination of co-regulators such as B7 proteins, which consist of co-stimulatory and co-inhibitory molecules expressed on antigen-presenting cells (APC), with their corresponding receptors on T cells. Co-stimulation is balanced by co-inhibitory signals, which ultimately determines whether the T cell response is activating or inhibitory.¹

The B7 family of immunoglobulins has ten reported members, which function as important secondary signals to either co-stimulate or co-inhibit T cells by selectively binding to T cell ligands, with B7-H2, B7-H6 and B7-H7 stimulating activation of naïve T cells;²⁻⁴ B7-DC, B7-H4 and B7-H5 inhibiting T cell responses;⁵⁻⁷ and B7-1 and B7-2 providing both stimulatory and inhibitory signals.⁸⁻¹⁰ Under normal circumstances, co-stimulators facilitate the development of protective immunity via receptors such as CD28 and CD28H,^{9, 11} whereas co-inhibitors including CTLA-4 and PD-1 inhibit inflammation to avoid over-activation.¹²

Co-inhibitory signaling pathways, including B7/CTLA-4 and PD-L1(B7-H1)-PD-1,

have over the past decade proven to be important targets in cancer immune checkpoint therapy,¹³ as evidenced by the clinical success of anti-CTLA-4 and anti-PD-1 antibodies (Ab) in various cancers. Drugs like atezolizumab that target PD-L1, which is highly expressed in many cancers, have been effective in clinical trials in the treatment of several different cancers. The B7 family and their receptors therefore have great potential as therapeutic targets in cancer immune checkpoint therapy.

In this study we used existing, publically available data from cBioPortal, to investigate various aspects of B7 family members and their corresponding receptors in various cancers. Based on these results we selected breast cancer for further analyses of B7 gene alterations, mRNA expression and DNA methylation as well as relevant clinical data.

We identified specific B7 family members with therapeutic and prognostic potential and report several putative ligands or targets of the B7 family with so far unknown receptors through network analysis.

Results

B7 family co-signaling molecules

To date, ten B7 family proteins have been identified, which, unfortunately, have not been consistently named between studies. We therefore propose a unified nomenclature by using the gene names as listed in NCBI (**Fig. 1A**). The ten members of the B7 family: B7-1, B7-2, B7-DC, B7-H1, B7-H2, B7-H3, B7-H4, B7-H5, B7-H6 and B7-H7 are mainly expressed by APCs and tumor cells. B7 proteins function as secondary signals that co-stimulate or co-inhibit T-cells by selectively binding to

CD28 proteins expressed on T-cells (**Fig. 1B**). Alignment of B7 family amino acid sequences indicated that each of the ten B7 proteins shared at least 15% identity with all other B7 family members (**Supplementary Table 1**). To further analyze the relationship among B7 family members, a phylogenetic tree was constructed (**Fig. 1C**). Our phylogenetic tree of human B7 was generated using MEGA and was divided into three groups, which shows that B7-H7 is the least similar to any of the other proteins (B7-1, B7-2, B7-DC, B7-H1, B7-H2, B7-H3, B7-H7, B7-H4, B7-H6) with high bootstrap probability.

Determination of B7 family gene alterations across different cancers

The frequencies of B7 family gene alterations (including mutations, deletions and amplifications) across various cancers are shown in **Fig. 2**. B7 mutations and deletions were less frequent than amplifications in cancer patients. B7 family genes were notably amplified in several cancers, including breast, lung, ovarian and stomach cancer. In breast cancer, all ten B7 proteins were amplified, with no mutations in 29 of the patients; in this dataset more than 50% of cases had B7 amplifications, which suggests important roles for these proteins in breast cancer.

B7 family proteins are over-expressed in breast cancer

Given the high frequency of B7 gene amplification in breast cancer, B7 expression was likely also dysregulated. We therefore assessed B7 genetic alterations in breast cancer data from TCGA, queried with cBioPortal.¹⁴ For each of the ten B7 genes, mutations were either not observed in any of 1098 breast cancer patients, or present at less than 1% in the case of B7-2 and B7-H2 (**Fig. 3A**). Interestingly, B7 mRNA levels

were up-regulated in about 10% (~100/1098) of breast cancer cases. Notably, B7-1, B7-DC, B7-H4, B7-H6 and B7-H7 were exclusively up-regulated with no down-regulation in any of the cases (**Fig. 3B**). We also evaluated B7 family expression in a cohort of 82 patients with triple-negative breast cancer, which has limited therapeutic options; several B7 family members were up-regulated to a greater extent in triple-negative breast cancer compared with the full cohort of 1098 patients with all types of breast cancer. B7-H3 was the only B7 protein that was down-regulated in (only 2/82) triple-negative breast cancer cases (**Fig. 3C**).

Although B7 proteins are constitutively expressed in breast cancer,¹⁵ the underlying mechanism thereof remains unknown. We therefore investigated the correlation between B7 promoter DNA methylation with their mRNA expression levels. Intriguingly, B7-1, B7-2, B7-DC, B7-H3, B7-H4 and B7-H5 mRNA levels were negatively correlated with promoter methylation status in the 1098 breast cancer cases (**Fig. 4A**) as well as in the 82 patients with triple-negative breast cancer (**Fig. 4B**). These results indicate that B7 family expression may be epigenetically regulated in breast cancer.

B7-H6 as a potential prognostic biomarker in breast cancer

Overall survival was compared between tumor samples with or without alterations in each of the B7 family members using cBioPortal. Notably, patients with B7-H6 alterations had significantly worse overall survival (**Fig. 5A**). We also determined whether B7-H6 was altered in four different data sets from cBioPortal, and found B7-H6 gene amplifications in each of the four data sets, albeit at varying frequencies

(**Fig. 5B**). Meanwhile, B7-H6 expression was associated with positive surgical margins (**Fig. 5C**), which suggests that B7-H6 expression may be associated with severe local invasion. These results suggest that B7-H6 may be a promising target for breast cancer immunotherapy. Finally, to elucidate the underlying mechanism of B7-H6 overexpression, mRNA levels (which were not significantly correlated with promoter methylation) were compared by B7-H6 gene alteration (shallow deletions, diploid, copy number gains, amplifications) in cBioPortal. Not surprisingly, B7-H6 mRNA was increased in cases with B7-H6 relative to all the other groups (**Fig. 5D**).

Network analysis of B7 family proteins in breast cancer

The genomic alterations in B7 family signaling networks in breast cancer are listed in **Fig. 6A**. CD28, PD1 and CTLA4, ICOS were amplified together with their ligands B7-1, B7-2, B7-H1 and B7-H2. Interestingly, we identified four putative B7 receptors within this network—MARCH8 (Membrane-associated RING-CH 8), BTLA (B and T lymphocyte attenuator), SMAD3 and PLSCR1 (Phospholipid Scramblase 1)—that had similar genomic alterations to B7-2, B7-H4 and B7-H5, respectively. We are the first to report these putative interactions (B7-2/MARCH8, B7-H4/BTLA, B7-H5/SMAD3, B7-H5/PLSCR1). Alignment of the amino acid sequences of MARCH8, BTLA, SMAD3 and PLSCR1 to other CD28 family members indicates that these proteins share at least 10% identity with all other CD28 family members (Supplementary Table 2). Phylogenic analysis further revealed that MARCH8 and PLSCR1 formed an exclusive group, while SMAD3 clustered with ICOS, CTLA-4 and CD28, and BTLA clustered with NKp30, PD-1 and TMIGD2 (**Fig. 6B**).

Domain analysis showed that N-terminal signaling peptides, V-set domains and transmembrane regions were present in most CD28 molecules (**Fig. 6C**). The newly identified B7-H4-interacting protein BTLA has domains similar to that of the CD28 family, which indicates that BTLA may well be a novel B7-H4 receptor. MARCH8, PLSCR1 and SMAD3 do not contain any v-set domains, suggesting that these proteins may be involved in T cell signaling under the regulation of B7 family.

Discussion

Co-stimulation comprises a complex molecular network principally including the B7 and CD28 protein families, which modulate T cell responses. Recent efforts have led to the identification of novel B7 family members and their corresponding receptors, which has deepened our understanding of their role in the immune system and in cancer. Given the promising results obtained with second generation antibodies like anti-PD-L1 in the treatment of advanced, treatment-refractory cancers in recent clinical trials, the B7 family of co-inhibitory molecules are being closely watched as potential immune checkpoint cancer therapeutic targets, particularly B7-H5 and B7-H7.^{7, 10, 11} Here, we provide an overview of the ten currently known B7 family members, under a unified nomenclature based on previous studies. Our phylogenetic tree of human B7 was generated using MEGA and was divided into three groups: B7-H1 and B7-DC (which is consistent with reports from Zhao et al.¹⁶); B7-H5; and the remaining members as the third group. Different phylogenetic classifications could be ascribed to different clustering algorithms. Additionally, there has been some confusion in the literature regarding the naming of B7-H5 and B7-H7.^{11, 17} Here, we

present a unified nomenclature of the B7 family according to the NCBI database.

Several B7 family members, including B7-H1, B7-H3 and B7-H4, are over-expressed in cancer, and their expression is significantly associated with cancer progression and prognosis.¹⁸⁻²⁴ We assessed genomic alterations in the ten B7 family members across multiple cancer types using cBioPortal and TCGA. B7 family members showed higher levels of amplification in lung and ovarian cancer, but particularly in breast cancer, with more than 50% of patients having at least one B7 family gene amplification. We therefore analyzed B7 family expression in 1098 patients with breast cancer and 82 patients with triple-negative breast cancer. Consistent with the amplification results, most B7 family members had increased levels of mRNA expression at varying frequencies, while a few members (including B7-2, B7-H2, B7-H3 and B7-H5) were downregulated in a small proportion of breast cancer patients. Among triple-negative breast cancer cases, only B7-H3 was downregulated, in two cases.

Intriguingly, the results of DNA methylation analysis showed that the expression of B7-1, B7-2, B7-DC, B7-H3, B7-H4 and B7-H5, but not that of B7-H1, B7-H2, B7-H6 and B7-H7, were negatively correlated with methylation of their respective promoters in both breast cancer and triple-negative breast cancer. Taken together with the gene amplification results (**Fig. 2**), we speculate that the expression of B7-1, B7-2, B7-DC, B7-H3, B7-H4 and B7-H5 might be regulated by both gene amplification and promoter methylation, while the expression of B7-H1, B7-H2, B7-H6 and B7-H7 might be more strongly regulated by gene amplification.

Of the B7 family, only B7-H6 alterations (primarily gene amplifications) were significantly associated with worse overall survival in a large cohort of breast cancer patients. Moreover, B7-H6 expression was associated with positive surgical margins and was decreased in breast cancer patients after postoperative radiotherapy. However, other studies have reported that B7-H6 has limited value as a prognostic marker in lung and gastric cancers.^{25, 26} Interestingly, however, Zhou et al. found that overall survival was significantly improved in ovarian cancer patients with relatively lower B7-H6 expression.²⁷ Semeraro M et al demonstrated that serum concentration of soluble B7-H6 correlated with the down-regulation of NKp30, bone marrow metastases, and chemoresistance of high-risk neuroblastoma (HR-NB), and they also indicated that soluble B7-H6 contained in the serum of HR-NB patients inhibited NK cell functions *in vitro*.²⁸ Together, our results support a notorious role of B7-H6 in the progression of certain types of cancer. Hence, we conclude that the outcome of B7-H6 expression might vary by cancer type. B7-H6 reportedly alerts the innate immune system to cellular transformation by binding its corresponding receptor, NKp30, on natural killer cells.⁴ B7-H6 has consistently been detected in various cancers but not in normal tissues.^{4, 29} It was recently reported that defective expression and function of NKp30 might be induced by B7-H6 in ovarian cancer, which results in impaired NK cell-dependent interferon-gamma (IFN γ) production and cytolytic function. B7-H6-mediated downregulation of NKp30 in NK cells might therefore contribute to immune evasion in ovarian cancer.³⁰ Although this mechanism has not been investigated in breast cancer, it may well explain the clinical significance of increased

B7-H6 expression in breast cancer, since B7-H6 is involved in the progression of human breast cancer and could function as a significant prognostic biomarker.

The B7-CD28 family is the major driving force of T cell co-stimulation and co-inhibition. Manipulation of co-stimulatory or co-inhibitory checkpoints facilitates reversal of tumor-induced T-cell anergy observed in many cancers. Some of these family members have been well studied for their clinical impact on tumors, especially with regard to PDL1 (B7-H1)-PD1 and CD80(B7-1)/CD86(B7-2)-CTLA4. Immune checkpoint therapy with monoclonal antibodies directed at CTLA-4, PD-1, and PD-L1 has emerged as a successful treatment for advanced melanoma.³¹⁻³³ Other ligands or receptors of the B7-CD28 family have subsequently been investigated, with B7-H1,³⁴ B7-H3,^{35,36} B7-H4,³⁷ B7-H5,³⁸ B7-H6^{27,39} and B7-H7^{17,40} found to be aberrantly expressed in various cancers. However, B7 receptors are still poorly studied. Although the B7-H7 receptor was recently identified,¹⁷ other B7 receptors, including B7-H3, B7-H4, B7-H5, remain unknown. Using the network derived from cBioPortal, which consists of pathways and interactions from several databases, we identified BTLA as a putative receptor for B7-H4, which is consistent with a previous report.⁴¹ Moreover, BTLA is co-expressed with certain B7 family members including B7-H4.⁴² However, almost none of the relevant review articles include BTLA as a B7-H4 receptor.^{10,43-50} Our findings provide additional support for the role of BTLA as a B7-H4 receptor.

In summary, all ten B7 family members were overexpressed in breast cancer, at varying frequencies, and their upregulation is plausibly related to gene amplification

and/or DNA methylation. Importantly, only B7-H6 gene amplification was significantly associated with worse overall survival in breast cancer patients.

Methods

Bioinformatics analyses

The NCBI database was queried for human B7, CD28 and B7-interacting proteins (Supplementary File 1); their corresponding sequences were analyzed by SMART and CBS to locate protein domains, including signaling peptides, IgV-like domains and transmembrane regions. Amino acid sequences were analyzed for similarity with other known sequences by BLAST and multiple sequence alignments were generated using CLUSTALW. The protein family signature was identified by InterPro.⁵¹ A phylogenetic tree was constructed based on the full-length amino acid sequences of B7 family proteins and their corresponding ligands using the neighbor-joining algorithm within MEGA version 3.1.⁵²

Determination of B7 family alterations across different cancers

The frequency of B7 family gene alterations (including mutations, deletions, copy number gains and amplifications) were assessed across multiple cancer types using the cBioPortal for Cancer Genomics database and TCGA which contains 105 common cancer studies with almost 23000 patients' details.

Genetic and clinical profiles of B7 family proteins in breast cancer

Genomic alterations, including mRNA expression and promoter methylation were assessed across 1105 sequenced breast cancer samples from cBioPortal, which had clinical profiles, including survival data.

Network analysis

To identify genomic alterations in B7 family signaling networks in breast cancer, we used all B7 family members as query genes in cBioPortal and explored the resulting network, which was based on pathways and interactions from various databases including Human Reference Protein Database (HPRD), Reactome, National Cancer Institute (NCI), and the Memorial Sloan-Kettering Cancer Center (MSKCC) Cancer Cell Map.¹⁴

Statistical analyses

GraphPad Prism 5 (GraphPad Software, La Jolla, CA) was used for statistical analyses. Student's t-test (two-tailed) was used to compare the means between two groups. Overall survival data are displayed as Kaplan-Meier plots with P-values calculated using the log-rank test. mRNA expression data were presented as mean \pm SD. P-values less than 0.05 were considered statistically significant.

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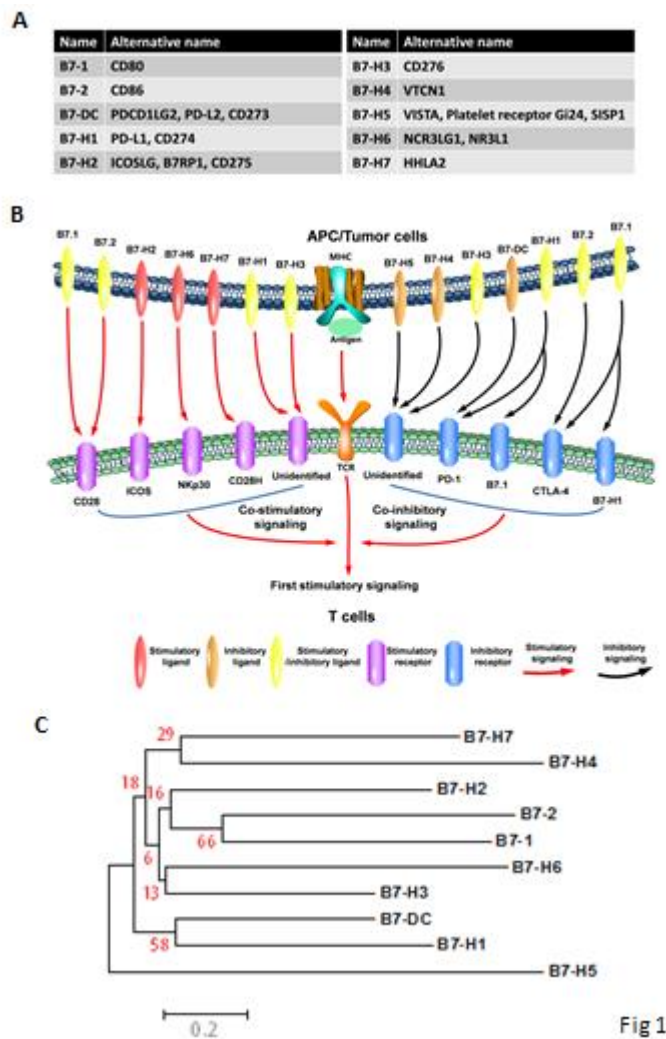


Fig 1

Figure 1. B7 family co-signaling molecules. **A)** Unified nomenclatures of each B7 family member. **B)** Interactions between co-stimulatory and co-inhibitory B7 and CD28 family members. **C)** Phylogenetic analysis of B7 family members. Neighbor-joining tree was constructed with Mega3.1 program. The numbers on the branches represent the confidence level of 10,000 bootstrap replications.

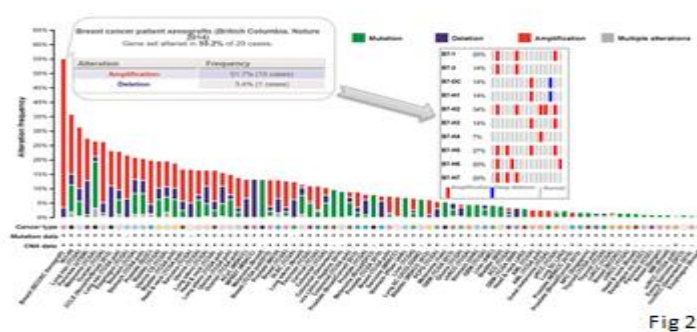


Fig 2

Figure 2. The alteration frequencies of B7 family across 105 different cancer studies. B7 family amplification frequencies are the highest in breast cancer. For the alteration frequencies of B7 family across 105 different cancer sets, the red bars indicate gene amplifications, blue bars are homozygous deletions, green bars are nonsynonymous mutations, gray bars indicate multiple alterations. For the alteration frequencies of B7 family in breast cancer, red bars indicate gene amplifications, blue bars are homozygous deletions, gray bars indicate no changes.



Figure 3. The mRNA expression of B7 family are up-regulated in breast cancer.

A) Mutation alterations of B7 family in 1098 breast cancer cases. **B)** The dysregulation of B7 family mRNA levels in 1093 breast cancer cases. **C)** The dysregulation of B7 family mRNA levels in 82 patients with triple-negative breast cancer. Green bars indicate gene mutations, red bars indicate mRNA up-regulation, blue bars indicate mRNA downregulation.

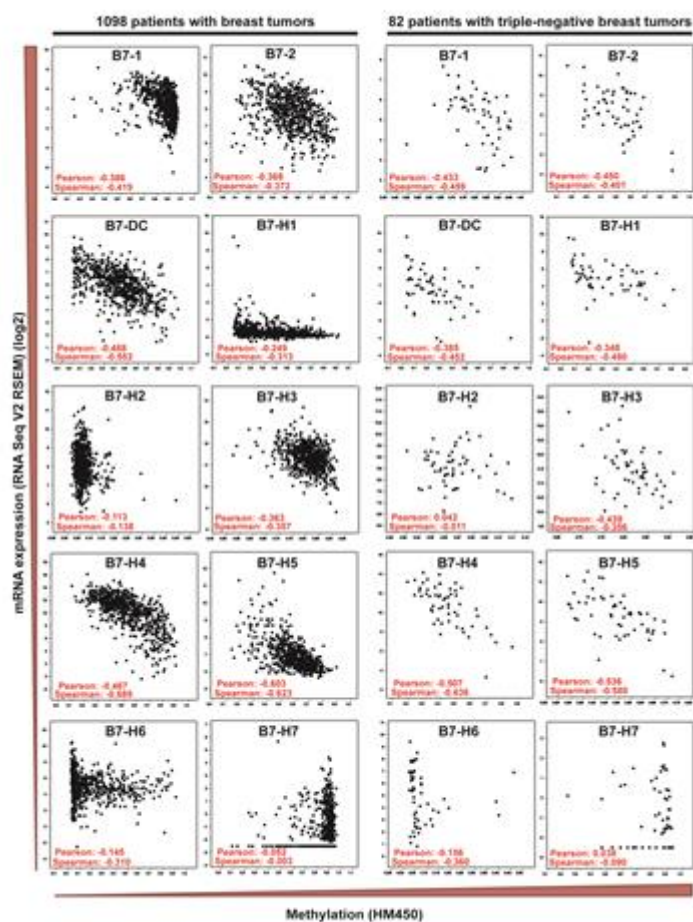


Fig 4

Figure 4. The correlation between the promoter DNA methylation status and the mRNA expression levels of B7 family in breast cancer. **A)** The mRNA levels of B7-1, B7-2, B7-DC, B7-H3, B7-H4 and B7-H5 are negative correlated with their promoter methylated status respectively in 1098 breast cancer cases. **B)** The mRNA levels of B7 family are negative correlated with their promoter methylated status respectively in 82 patients with triple-negative breast cancer.

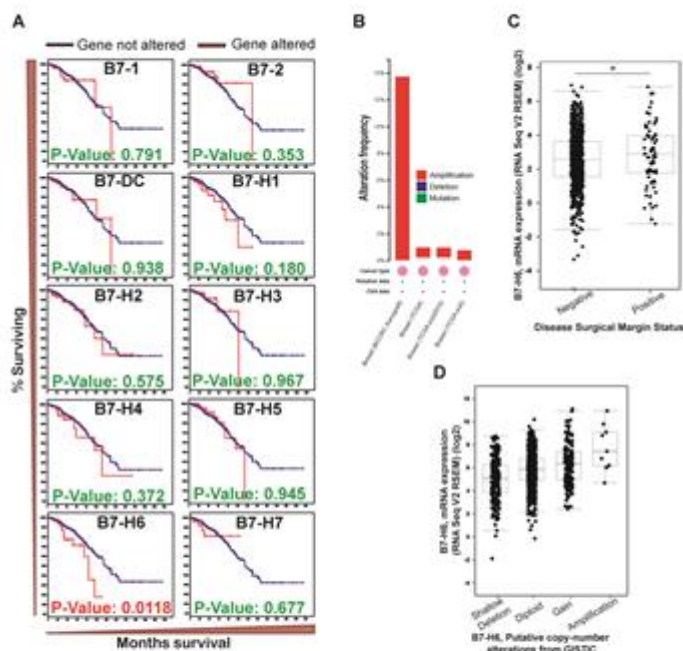


Fig 5

Figure 5. B7-H6 is a potential biomarker in breast cancer. **A)** The overall survival of breast cancer patients with B7 family alteration. **B)** The genetic alteration of B7-H6 in four different data sets from cBioPortal. **C)** The higher expression of B7-H6 in positive surgical margin when compared that in negative surgical margin. **D)** B7-H6 mRNA was increased in the breast cancer tissues in which B7-H6 was amplified.

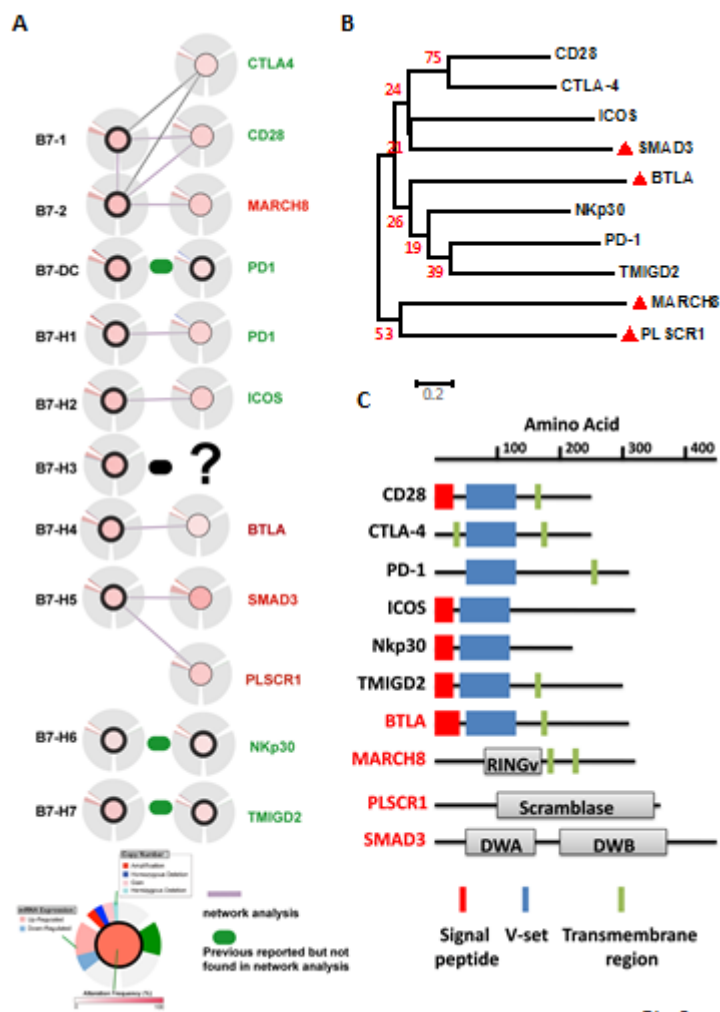


Fig 6

Figure 6. Network analysis of B7 family networks in breast cancer. A) The genomic alterations in B7 family signaling networks in breast cancer. **B)** Phylogenetic analysis of B7 reacting proteins. Neighbor-joining tree was constructed with Mega3.1 program. The numbers on the branches represent the confidence level of 10,000 bootstrap replications. **C)** The domain analysis of B7 family reacting proteins.