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Critical Immune Checkpoints Linked with NK and T Cells for Overall Survival of Breast Cancer Subtypes

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Abstract: Breast cancer is the second leading cause of cancer death in women. Since cancer disrupts immune checkpoints to suppress the anti-tumor response, we assessed immune checkpoint signatures linked with NK and T cells in breast cancer including triple-negative breast cancer (TNBC) subtypes. Furthermore, critical immune checkpoints related to overall survival were identified using the in-silico and comparative analysis. Immune checkpoint signatures were breast cancer subtype-specific, showing differential signature in each subtype. High levels of immune checkpoints were related to overall survival in some breast cancer subtypes. The differential overall survival rates of breast cancer subtypes may be due to the final net balance of total immune checkpoints by exerting either inhibitory or stimulatory interaction with immune cells. Critical immune checkpoints for poor overall survival of breast cancer subtypes are as follows: UL16 binding protein 2 (ULBP2) in both basal-like breast cancers and basal-like 2 TNBC subtype; V-set domain containing T cell activation inhibitor 1 (VTCN1) in immunomodulatory TNBC subtype. In conclusion, specific immune checkpoints may differentially influence overall survival in a breast cancer subtype-specific manner.

Keywords: immune checkpoints; NK cells; T cells; overall survival; breast cancer

1. Introduction

Immune checkpoints are regulators of the immune system which comprise diverse receptors and ligands, including stimulatory and inhibitory molecules, to maintain immune homeostasis [1,2]. Cancer disrupts these immune checkpoints, resulting in suppression of the anti-tumor immune response. Immunotherapy appears as a standard treatment in cancers, including immune checkpoint blockade and adoptive cell transfer [3]. Immune checkpoint blockade blocks inhibitory signals of immune cell activation, contributing to cancer treatment [4]. Natural killer (NK) cells belong to the innate immune system and contribute to antitumor immune responses [5]. The infiltration and cytotoxicity of NK cells in tumor tissues influence the survival of patients with cancer [6]. Immune checkpoint molecules and receptors display multiple inhibitory and stimulatory pathways, of which net balance leads to regulation of NK cell function in the tumor microenvironment. T cells, which belong to the adaptive immune system, play a key role in tumor surveillance [7]. Cancer can lead to the induction of T cell exhaustion which are characterized by an increased expression of inhibitory pathways and a loss of function [8]. Different CD4 T cell subsets in cancer can affect tumor immune responses [3] and cancer cells impede the cytotoxicity to CD8 T cells [7]. Cancer cells expressing specific immune checkpoint ligands may affect immune cells like NK and T cells, evading anti-tumorigenicity of immune cells. Cancer stem cells have immunomodulatory capabilities that protect cancer cells from immune clearance by producing immune system inhibitory factors [9].

Breast cancer is the most common type of cancer in women. Breast cancer subtypes are classified by the following immunohistochemical features: estrogen receptor (ER) and/or progesterone receptor (PR) positive (+) and human epidermal growth factor receptor 2 (HER2) negative (–) luminal A; ER and/or PR+ and HER2+ luminal B; ER–, PR– and HER2+ HER2-enriched (HER2); ER–, PR– and HER2– basal-like subtypes [10]. Triple-negative breast cancer (TNBC), which is characterized by the absence of ER, PR, and HER2 expressions, results in aggressive tumorigenicity and high mortality due to a lack of therapeutic targets compared with other breast cancer subtypes [11]. Basal-like breast cancer and TNBC are interchangeable based on facts that 71% of TNBC are found

to be basal-like while 77% of basal-like breast cancer are triple negative [12]. Both basal-like breast cancer and TNBC are associated with aggressive pathologic features and poor clinical outcomes [13]. TNBC is heterogeneous, including several subtypes with different molecular characteristics as follows: basal-like 1 and 2, immunomodulatory, mesenchymal, mesenchymal stem-like, and luminal androgen receptor subtypes [14]. Basal-like 1 subtype is enriched in cell cycle pathways, basal-like 2 subtype displays growth factor signals. Immunomodulatory subtype is enriched in immune cell signaling, mesenchymal and mesenchymal stem-like subtypes are enriched in epithelial-mesenchymal transition, and luminal androgen receptor subtype includes androgen receptor signaling [14]. A better understanding of intrinsic and clinical variations among TNBC subtypes may offer better options for therapeutic strategies, improving clinical outcomes with increased survival rates. Interestingly, immune checkpoints can promote chemoresistance in specific breast cancer cell lines [15], indicating the decreased survival of breast cancer patients.

To date, there have been no comprehensive reports on overall survival of breast cancer patients based on the immune checkpoint signatures in breast cancer subtypes. Here, we evaluated immune checkpoint signatures linked with NK and T cells in breast cancer including TNBC subtypes and how the signatures correlate with breast cancer survival. The identification of immune checkpoint molecules in breast cancer subtypes and the immune checkpoint-based overall survival provide a better understanding to restore the suppressed anti-tumor immunity. This understanding guides the selection of specific immune checkpoint inhibitors in a subtype-dependent manner for breast cancer treatment.

2. Materials and Methods

2.1. In Silico Data Analysis

Data analysis was performed on publicly available microarray datasets that were deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/, accessed on 25 January 2019) database under accession number GSE12777 for breast cancer cell lines (9 basal-like TNBC; 6 mesenchymal TNBC; 7 luminal androgen receptor TNBC; 11 HER2; 12 luminal A; 5 luminal B cell lines). We utilized Gitools 2.3.1 (http://www.gitools.org/, accessed on 27 January 2021) based on Oracle Java 7, an open-source tool to perform Genomic Data Analysis and Visualization as interactive heat-maps [16]. Kaplan-Meier plotter database (https://kmplot.com/analysis/, accessed on 3 August 2021) was utilized to evaluate overall survival using proportional hazards regression to estimate hazard ratios (HRs) and 95% confidence intervals (CIs) based on gene expression profile of immune checkpoints among 1879 breast cancer patients from GEO and The Cancer Genome Atlas (TCGA); immune checkpoints were specified with probe sets (Affymetrix HG-U133A, HG-U133A 2.0, and HG-U133 Plus 2.0 microarrays) [17].

2.2. Statistical Analysis

Data were expressed as mean ± standard error of the mean (SEM) and analyzed by the Student's *t*-test and one-way analysis of variance (ANOVA) to detect statistical significance ($p < 0.05$). If statistical significance ($p < 0.05$) was indicated by ANOVA, then data were further analyzed using Tukey's pairwise comparisons to detect the specific group differences.

3. Results

3.1. Proposed Immune Checkpoints Linking Breast Cacner and Innate NK and Adaptive T Cells in the Tumor Microenvironment

Cancer cells escape immune surveillance through immunosuppression and the net balance between co-stimulatory and co-inhibitory molecules in immune cells plays a critical role in tumor-induced immunosuppression [18]. First, we summarized immune checkpoints linking breast cancer and immune cells, such as NK and T cells, through multiple inhibitory and stimulatory pathways (Figure 1). Breast cancer cells express immune checkpoint ligands to display inhibitory, both inhibitory and stimulatory, and stimulatory pathways by interacting with NK and T cells. Although some immune checkpoints of breast cancer are shared with both NK and T cells, NK and T cells form their specific immune checkpoints to respond to ligands secreted from breast cancer (Figure 1). The net balance of these multiple inhibitory and stimulatory pathways displays the final inhibitory and stimulatory response between breast cancer and immune cells.

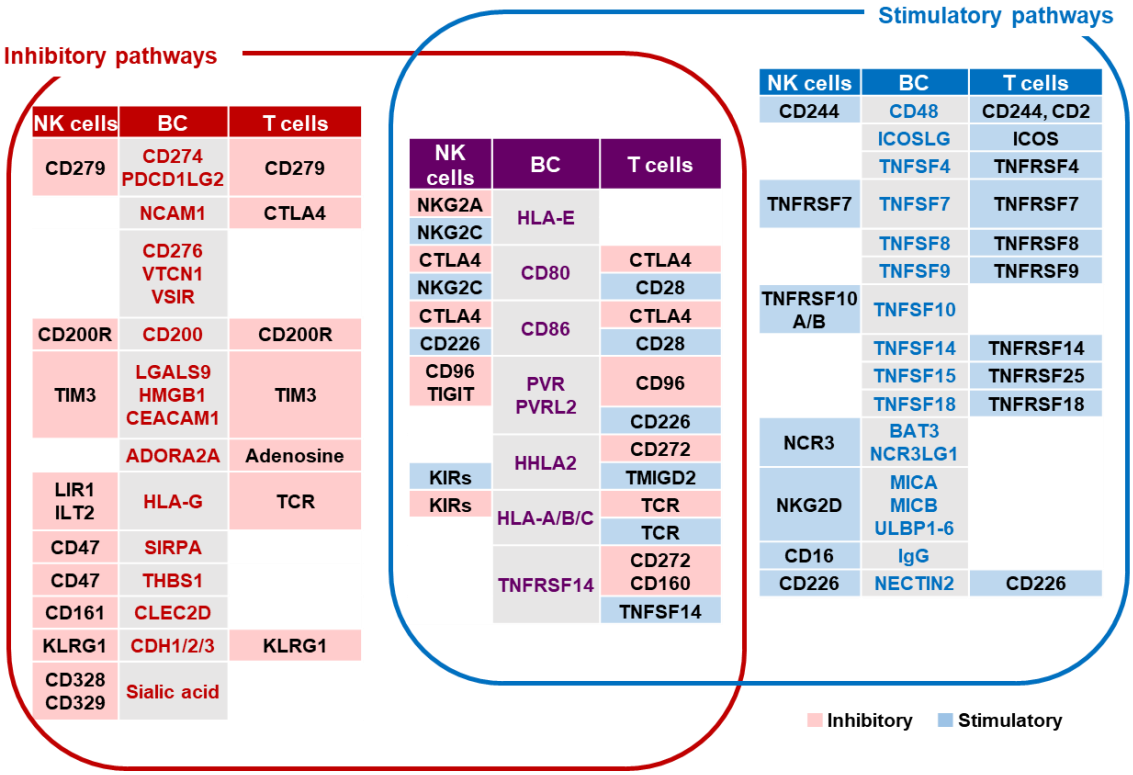

Figure 1. Immune checkpoints linking breast cancer (BC) and NK and T cells in the tumor microenvironment through multiple inhibitory and stimulatory pathways. Red, purple, and blue letters indicate inhibitory, inhibitory/stimulatory, and stimulatory pathways, respectively. CD279: cluster of differentiation 279 or programmed cell death 1 (PD-1); CD274: programmed death-ligand 1 (PD-L1) or B7 homolog 1 (B7-H1); PDCD1LG2: programmed cell death 1 ligand 2 (PD-L2); NCAM1: neural cell adhesion molecule 1; CTLA4: cytotoxic T-lymphocyte-associated protein 4 or CD152; CD276: B7 homolog 3 (B7-H3); VTCN1: V-set domain containing T cell activation inhibitor 1; VSIR: V-set immunoregulatory receptor; CD200R: CD200 receptor; LGALS9: galectin 9; HMGB1: high mobility group box protein 1; CEACAM1: carcinoembryonic antigen-related cell adhesion molecule 1 or CD66a; TIM3: T cell immunoglobulin and mucin-domain containing-3; ADORA2A: adenosine A2a receptor; HLA-G: human leukocyte antigen-G; LIR1: leukocyte immunoglobulin like receptor B1; ILT2: Ig-like transcript 2; TCR: T-cell receptor; SIRPA: signal regulatory protein alpha; CD47: cluster of differentiation 47 or integrin associated protein (IAP); THBS1: thrombospondin 1; CLEC2D: C-type lectin domain family 2 member D; CD161: killer cell lectin-like receptor subfamily B member 1 (KLRB1); CDH1/2/3: cadherin 1/2/3; KLRG1: killer cell lectin like receptor G1; CD328: sialic acid-binding Ig-like lectin 7 (SIGLEC7); CD329: sialic acid-binding Ig-like lectin 9 (SIGLEC9); HLA-E: human leukocyte antigen-E; NKG2A: killer cell lectin like receptor C1 (KLRC1); NKG2C: killer cell lectin like receptor C2 (KLRC2); PVR: poliovirus receptor or CD155; PVRL2: PVR-related 2 or CD112; CD96: T cell activation, increased late expression (TACTILE); TIGIT: T cell immunoreceptor with Ig and ITIM domains; HHLA2: human endogenous retrovirus-H long terminal repeat-associating protein 2; CD272: B And T lymphocyte associated (BTLA); KIRs: killer cell immunoglobulin-like receptors; TMIGD2: transmembrane and immunoglobulin domain containing 2; TNFRSF: tumor necrosis factor receptor superfamily; TNFSF: tumor necrosis factor superfamily; ICOSLG: inducible T cell costimulator ligand; ICOS: inducible T cell costimulator or CD278; BAT3: HLA-B-associated transcript 3; NCR3: natural cytotoxicity triggering receptor 3; NCR3LG1: NCR3 ligand 1; MICA: MHC class I polypeptide-related sequence A; MICB: MHC class I polypeptide-related sequence B; ULBP1-6: UL16 binding protein 1-6; NKG2D: natural killer group 2D; NECTIN2: nectin cell adhesion molecule 2.

3.2. Dominant Immune Checkpoints in Breast Cancer Subtypes from Breast Cancer Patients

Based on immune checkpoints linking breast cancer and NK and T cells (Figure 1), we evaluated immune checkpoint signatures in breast cancer subtypes from patients. Then we have compared expression levels of immune checkpoints in inhibitory, both inhibitory and stimulatory, and stimulatory pathways between breast cancer subtypes (Figure 2). Basal-like breast cancer showed high levels of NCAM1, VTCN1, HLA-A, HLA-G, SIRPA, and CDH3 for inhibitory pathways; PVR for inhibitory or stimulatory pathways; ULBP1 and ULBP3 for stimulatory pathways compared to other subtypes. Both basal-like and HER2 subtypes showed high levels of

CD274 and PDCD1LG2 for inhibitory pathways; HLA-B for inhibitory or stimulatory pathways; ICOSLG, TNFSF9, and ULBP2 for stimulatory pathways compared to other subtypes. Both HER2 and luminal B subtypes showed high levels of CDH1 for inhibitory pathways. Specific immune checkpoints were not found for HER2, luminal A, and luminal B subtypes at this point. Interestingly, basal-like breast cancer showed lower levels of PVRL2 for inhibitory or stimulatory pathways and TNFSF4 and TNFSF10 for stimulatory pathways compared to other subtypes (Figure 2).

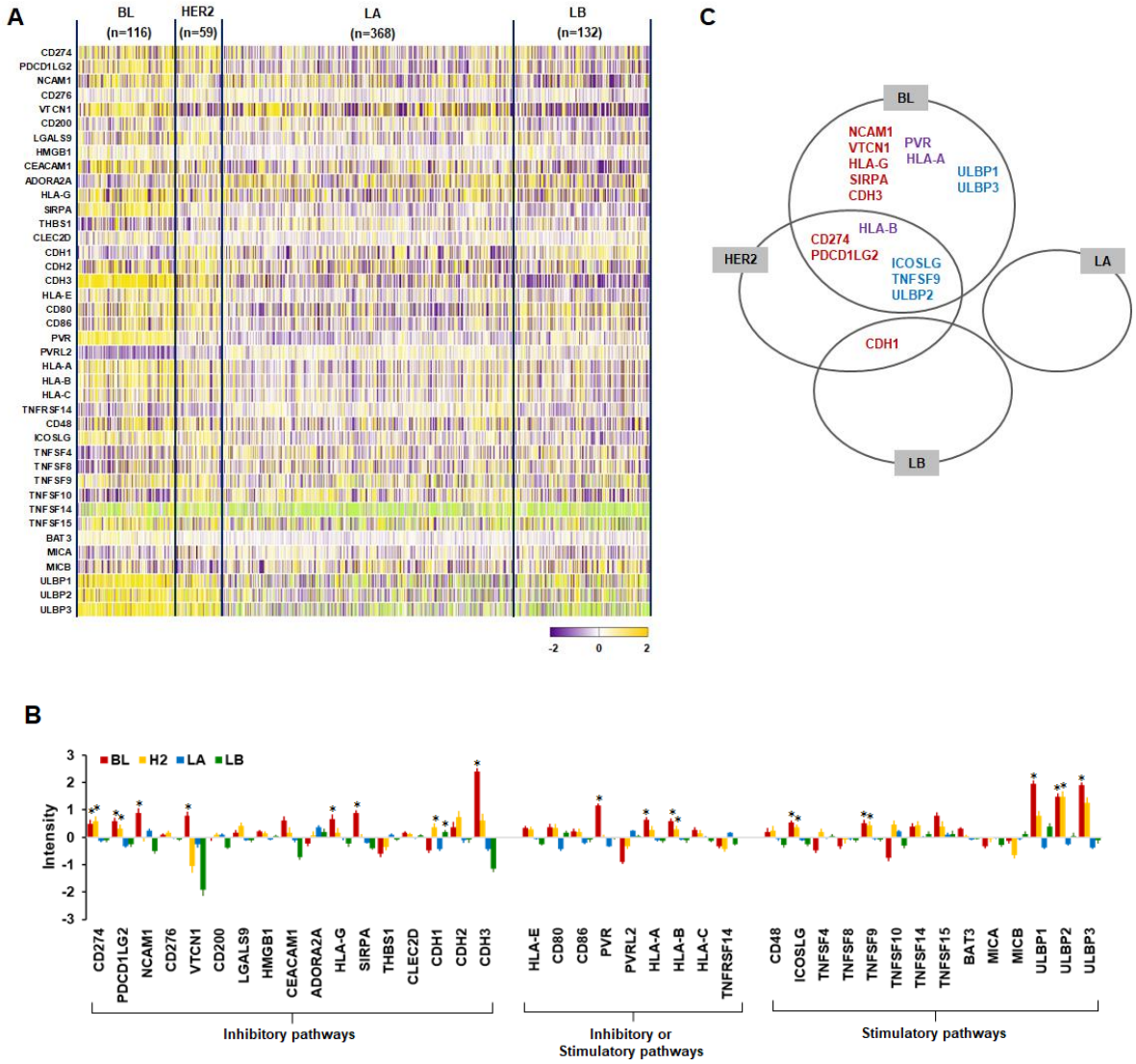

Figure 2. Immune checkpoint signature in breast cancer subtypes. (**A**) Heatmap of immune checkpoint expression profiles in breast cancer subtypes obtained from TCGA-based dataset using Gitools 2.3.1., including basal-like (BL, n = 116) with ER–, PR– and HER2–; HER2 (n = 59) with ER–, PR– and HER2+; luminal A (LA, n = 368) with ER and/or PR+ and HER2–; luminal B (LB, n = 132) with ER and/or PR+ and HER2+. (**B**) Statistical analysis of intensities from heatmap of immune checkpoint expressions in breast cancer subtypes. Red, yellow, blue, and green bars specify expression levels in BL-, HER2-, LA- and LB-breast cancer subtypes, respectively. * indicates dominant immune checkpoint expressions using ANOVA and Tukey's pairwise comparisons ($p < 0.05$). (**C**) Summary of inhibitory and/or stimulatory pathway-related immune checkpoints in breast cancer subtypes. Red, purple, and blue gene names indicate significant increases in inhibitory, inhibitory/stimulatory, and stimulatory pathways, respectively.

3.3. Dominant Immune Checkpoints in Breast Cancer Subtypes from Breast Cancer Cell Lines

We evaluated immune checkpoint signatures in basal-like TNBC, mesenchymal TNBC, luminal androgen receptor TNBC, HER2, luminal A, and luminal B breast cancer cell line subtypes. Then we have compared expression levels of immune checkpoints in inhibitory, both inhibitory and stimulatory, and stimulatory pathways between specific-type breast cancer cell lines (Figure 3A–C). Basal-like TNBC showed high levels of HLA-G and

CDH3 for inhibitory pathways; HLA-A, HLA-B, and HLA-C for both inhibitory and stimulatory pathways (Figure 3A,B). Mesenchymal TNBC showed high levels of CDH2 for inhibitory pathways and MICB for stimulatory pathways (Figure 3A,C). Luminal A subtype had high levels of CDH1 for inhibitory pathways and both luminal A and B subtypes showed high levels of PVRL2 for both inhibitory and stimulatory pathways (Figure 3A,B). To exclude tumor heterogeneity, we investigated the intersection of immune checkpoint signatures between human breast cancer tumors (Figure 2) and cell lines (Figure 3). HLA-A, HLA-B, HLA-G, and CDH3 were highly expressed in both human basal-like tumors and TNBC cell lines (Figure 3D).

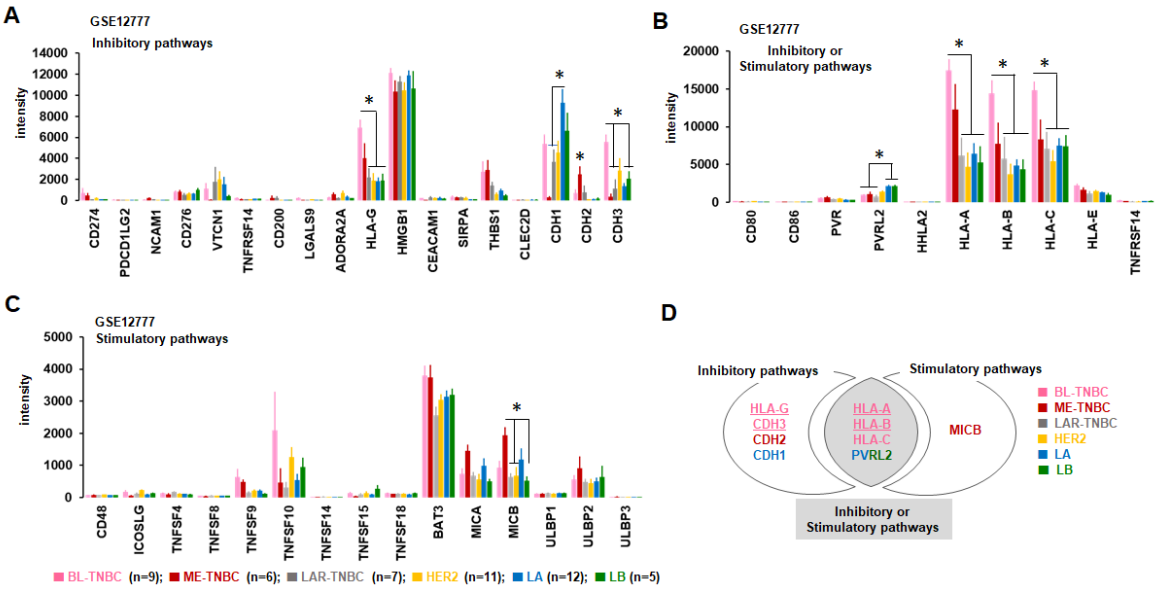

Figure 3. Immune checkpoint signature in breast cancer cell lines. Comparisons for RNA expression levels of (**A**) inhibitory pathway-, (**B**) inhibitory or stimulatory pathway-, and (**C**) stimulatory pathway-related immune checkpoints based on analysis of NCBI GEO dataset (Accession: GSE12777) with 50 human breast cancer cell lines using Gitools 2.3.1. Pink, red, gray, yellow, blue, and green bars specify expression levels in basal-like (BL) TNBC, mesenchymal (ME) TNBC, luminal androgen receptor (LAR) TNBC, HER2, luminal A (LA), and luminal B (LB) subtype breast cancer cells, respectively. * indicates significance compared with dominant immune checkpoint expressions using the Student's *t*-test ($p < 0.05$). (**D**) Summary of significant immune checkpoint signatures in breast cancer subtypes based on cell lines. Underlines indicate immune checkpoints obtained from intersection between human breast cancer tumors and cell lines to exclude the tumor heterogeneity. Breast cancer cell lines were used as follows: HCC1143, HCC1937, HCC38, MDA-MB-468, CAL85-1, DU4475, HCC1806, HCC70, and HDQ-P1 cells for BL-TNBC; CAL-120, CAL-51, BT-549, Hs578T, MDA-MB-231, and MDA-MB-436 cells for ML-TNBC; BT20, CAL-148, HCC1395, MDA-MB-453, MFM-223, MX1, and SW527 for LAR-TNBC; AU565, EVSA-T, HCC1419, HCC1569, HCC1954, HCC202, HCC2218, JIMT-1, KPL4, SKBR3, and UACC-893 cells for HER2; BT483, CAMA-1, EFM-192A, HCC1428, HCC1500, KPL1, MCF7, MDA-MB-134VI, MDA-MB-175VII, MDA-MB-415, T47D, and ZR75-1 cells for LA; BT474, EFM19, MDA-MB-361, UACC-812, and ZR75-30 cells for LB.

3.4. Overall Survival-Related Immune Checkpoints in Breast Cancer Patients

We evaluated overall survival of breast cancer patients based on immune checkpoint signatures using datasets for breast cancer subtypes (Table 1) and TNBC subtypes (Table 2). In breast cancer subtypes, basal-like subtype revealed improved overall survival with high levels of the following checkpoints: CD274, HLA-G, and CLEC2D for inhibitory pathways; CD80, CD86, HLA-A, HLA-B, HLA-C, HLA-E, and TNFRSF14 for inhibitory/stimulatory pathways; CD48, TNFSF14, and MICB for stimulatory pathways (Figure 4A). Interestingly, high levels of ULBP2 for stimulatory pathways were associated with poor overall survival in basal-like subtype (Figure 4A). HER2 subtype had an improved overall survival with high levels of CD86 for inhibitory/stimulatory pathways and luminal A subtype showed improved overall survival with high levels of VTCN1 for inhibitory pathways and TNFSF8 for stimulatory pathways (Figure 4A). Luminal B subtype showed improved overall survival with high levels of CD48 for stimulatory pathways but poor overall survival with THBS1 and CDH2 for inhibitory pathways and high levels of ULBP3 for stimulatory pathways (Figure 4A). In TNBC subtypes, basal-like 1 TNBC showed improved overall survival with high levels of CD274 for inhibitory pathways and with high

levels of CD48 and MICB for stimulatory pathways (Figure 4B). On the other hand, basal-like 2 TNBC had poor overall survival with high levels of CD200 and LGALS9 for inhibitory pathways, PVR for inhibitory/stimulatory pathways, and ULBP2 for inhibitory/stimulatory pathways (Figure 4B). Immunomodulatory TNBC showed poor overall survival with high levels of VTCN1 for inhibitory pathways but improved overall survival with high levels of PDCD1LG2 for inhibitory pathways and TNFRSF14 for inhibitory/stimulatory pathways (Figure 4B). Mesenchymal TNBC had poor overall survival with high levels of CDH1 for inhibitory pathways and improved overall survival with high levels of CD48 for stimulatory pathways (Figure 4B). Mesenchymal stem-like TNBC showed an improved overall survival with high levels of LGALS9 for inhibitory pathways and BAT3 and ULBP1 for stimulatory pathways (Figure 4B). Luminal androgen receptor TNBC had a poor overall survival with high levels of CD80 and HHLA2 for inhibitory/stimulatory pathways but an improved overall survival with high levels of CDH1 for inhibitory pathways (Figure 4B). Based on the relationship between immune checkpoints and overall survival in breast cancer and TNBC subtypes, ULBP2 in both basal-like breast cancer and basal-like2 TNBC and VTCN1 in immunomodulatory TNBC appeared as critical immune checkpoints for a poor overall survival in breast cancer (Figure 4C), as shown by HR 1.52 (95% CI 1.04–2.23), 3.23 (1.04–10.0), and 2.51 (1.10–5.73), respectively (Figure 4D).

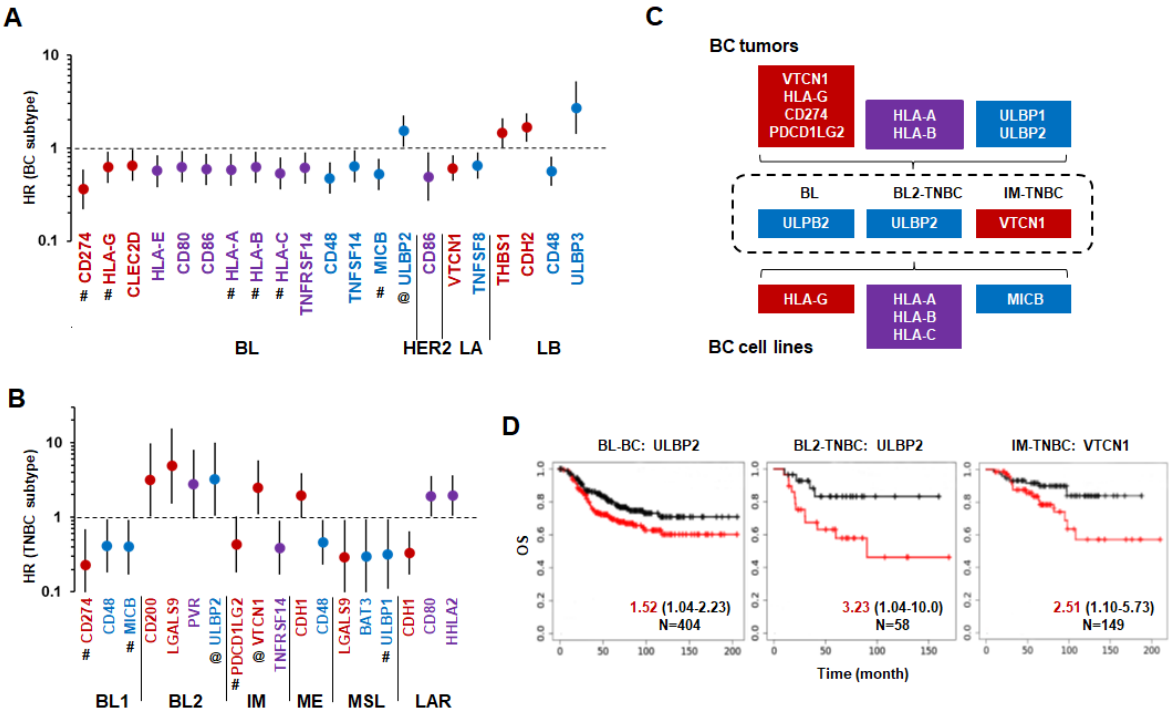

Figure 4. The overall survival-related immune checkpoints in breast cancer and TNBC subtypes. The HR of immune checkpoints that were either poor (above 1) or good (below 1) overall survival (OS) in (**A**) breast cancer (BC) and (**B**) TNBC subtypes using GEO and TCGA datasets available in the Kaplan-Meier plotter database (https://kmplot.com/analysis/, accessed on 3 August 2021). # and @ indicate dominant genes with good and poor OS, respectively, in breast cancer. Red, purple, and blue dots and letters indicate significant increases in inhibitory, inhibitory/stimulatory, and stimulatory pathways, respectively. (**C**) Intersection of the overall survival-related immune checkpoints obtained from immune checkpoint signatures between human breast cancer cell lines and tumors. Red, purple, and blue boxes indicate significant increases in inhibitory, inhibitory/stimulatory, and stimulatory pathways, respectively. (**D**) Kaplan-Meier plots for the survival of BL breast cancer, BL2-TNBC, and IM-TNBC patients with the ULBP2 and VTCN1 signatures, respectively. HRs and 95% CIs for overall survival of patients with breast cancer subtypes and immune checkpoint signatures indicated on the plots. Red numbers indicate statistically significant values of HRs ($p < 0.05$). The HRs were determined using the GEO and TCGA datasets available in the Kaplan-Meier plotter database (https://kmplot.com/analysis/, accessed on 3 August 2021). Black and red lines indicate low and high levels of targeted genes, respectively.

Table 1. Hazard ratio (HR) and 95% confidence interval (CI) for overall survival based on the expression levels of immune checkpoints in breast cancer subtypes.

		Basal-like		HER2		Luminal A		Luminal B	
Gene	**ID**	**HR**	**95% CI**	**HR**	**95% CI**	**HR**	**95% CI**	**HR**	**95% CI**
CD274	227458_at 223834_at	**0.36**	0.22–0.59	0.53	0.26–1.08	0.92	0.57–1.49	0.61	0.33–1.14
PDCD1LG2	220049_s_at	0.77	0.53–1.13	0.60	0.33–1.08	0.92	0.67–1.26	0.84	0.59–1.19
NCAM1	209968_s_at 212843_at 214952_at 217359_s_at	1.21	0.83–1.78	0.95	0.54–1.67	1.00	0.73–1.37	1.17	0.82–1.66
CD276	224859_at 1552914_a_at	1.10	0.69–1.75	0.67	0.34–1.34	1.48	0.91–2.41	1.60	0.85–2.98
VTCN1	219768_at	1.02	0.70–1.49	0.67	0.37–1.19	**0.60**	0.44–0.83	0.92	0.65–1.30
CD200	209582_s_at 209583_s_at	1.03	0.71–1.51	0.89	0.51–1.57	0.85	0.62–1.17	0.76	0.53–1.08
LGALS9	203236_s_at	0.74	0.50–1.08	0.61	0.34–1.09	0.95	0.70–1.31	1.03	0.73–1.45
HMGB1	200679_x_at 200680_x_at 214938_x_at 216508_x_at	0.87	0.59–1.27	1.07	0.61–1.88	1.00	0.73–1.38	0.92	0.65–1.30
CEACAM1	206576_s_at 209498_at 210610_at 211883_x_at 211889_x_at	0.75	0.51–1.10	1.14	0.65–2.02	1.04	0.75–1.42	0.89	0.63–1.27
ADORA2A	205013_s_at	0.75	0.51–1.10	0.94	0.53–1.65	0.85	0.62–1.16	0.99	0.70–1.40
HLA-G	210514_x_at 211528_x_at 211529_x_at 211530_x_at	**0.62**	0.42–0.92	0.64	0.36–1.13	0.74	0.54–1.02	0.81	0.57–1.15
SIRPA	202895_s_at 202896_s_at 202897_at 217024_x_at	1.07	0.73–1.57	0.86	0.49–1.52	0.87	0.63–1.19	1.01	0.71–1.43
THBS1	201107_s_at 201108_s_at 201109_s_at 201110_s_at	1.41	0.96–2.07	1.16	0.66–2.04	1.22	0.89–1.68	**1.45**	1.02–2.07

	215775_at								
CLEC2D	220132_s_at	**0.65**	0.44–0.96	0.74	0.42–1.31	0.77	0.56–1.05	0.69	0.49–0.98
CDH1	201130_s_at 201131_s_at 209414_at 209415_at 209416_s_at 211865_s_at	1.19	0.82–1.75	1.51	0.85–2.68	1.09	0.79–1.49	1.28	0.90–1.81
CDH2	203440_at 203441_s_at	1.03	0.70–1.50	1.31	0.74–2.33	1.16	0.84–1.59	**1.67**	1.17–2.37
CDH3	203256_at 206327_s_at 206328_at	1.16	0.79–1.70	1.15	0.65–2.02	0.97	0.71–1.33	1.21	0.85–1.71
HLA-E	200904_at 200905_x_at 217456_x_at	**0.57**	0.38–0.84	0.78	0.44–1.38	1.02	0.75–1.40	0.75	0.53–1.07
CD80	207176_s_at	**0.63**	0.43–0.93	1.08	0.62–1.91	0.88	0.64–1.21	0.97	0.68–1.37
CD86	205685_at 205686_s_at 210895_s_at	**0.59**	0.40–0.87	**0.49**	0.27–0.89	0.95	0.69–1.30	0.81	0.57–1.15
PVR	212662_at 214443_at 214444_s_at 216283_s_at 32699_s_at	1.00	0.68–1.46	1.23	0.70–2.18	1.07	0.78–1.47	1.10	0.77–1.56
PVRL2	203149_at	1.27	0.86–1.86	0.85	0.48–1.50	1.09	0.79–1.50	0.74	0.52–1.05
HHLA2	220812_s_at	1.39	0.95–2.03	0.86	0.49–1.51	0.82	0.60–1.12	1.19	0.84–1.69
HLA-A	213932_x_at 215313_x_at	**0.58**	0.39–0.86	0.90	0.51–1.58	1.10	0.80–1.51	0.88	0.62–1.24
HLA-B	208729_x_at 209140_x_at 211911_x_at	**0.62**	0.42–0.91	0.84	0.48–1.49	0.99	0.72–1.36	1.06	0.75–1.49
HLA-C	208812_x_at 211146_at 211799_x_at 214459_x_at 216526_x_at	**0.53**	0.36–0.79	0.70	0.39–1.24	0.87	0.64–1.19	0.91	0.64–1.28
TNFRSF14	209354_at	**0.61**	0.41–0.89	1.14	0.64–2.01	0.90	0.66–1.24	0.80	0.57–1.14
CD48	204118_at	**0.47**	0.32–0.70	0.59	0.33–1.04	0.80	0.58–1.10	**0.56**	0.39–0.80

ICOSLG	211197_s_at 211198_s_at 211199_s_at 213450_s_at	1.24	0.85–1.82	0.91	0.52–1.61	0.79	0.57–1.08	0.94	0.66–1.32
TNFSF4	207426_s_at	0.75	0.51–1.09	0.64	0.36–1.14	1.13	0.82–1.55	1.15	0.81–1.63
TNFSF7	206508_at	0.79	0.54–1.15	0.91	0.51–1.60	1.05	0.76–1.44	0.88	0.62–1.25
TNFSF8	207216_at	1.25	0.86–1.83	0.93	0.53–1.64	**0.65**	0.47–0.89	0.97	0.69–1.38
TNFSF9	206907_at	0.86	0.59–1.26	1.48	0.83–2.64	0.82	0.60–1.13	1.22	0.86–1.73
TNFSF10	202687_s_at 202688_at 214329_x_at	0.69	0.47–1.01	0.90	0.51–1.59	0.91	0.66–1.25	0.91	0.64–1.29
TNFSF14	207907_at	**0.64**	0.43–0.94	0.62	0.35–1.10	0.89	0.65–1.21	1.06	0.75–1.51
TNFSF15	221085_at	0.83	0.57–1.21	0.84	0.47–1.48	1.06	0.77–1.45	1.16	0.82–1.65
TNFSF18	221371_at	1.00	0.69–1.47	1.00	0.57–1.76	1.10	0.80–1.51	1.21	0.85–1.71
BAT3	201255_x_at 210208_x_at 213318_s_at	0.70	0.48–1.02	0.60	0.34–1.07	1.25	0.91–1.71	1.17	0.82–1.65
MICA	205904_at 205905_s_at	1.16	0.80–1.70	1.27	0.77–2.25	0.82	0.59–1.13	1.33	0.93–1.88
MICB	206247_at	**0.52**	0.35–0.77	0.67	0.38–1.20	0.90	0.66–1.24	0.79	0.55–1.11
ULBP1	221323_at	1.13	0.77–1.65	1.51	0.84–2.69	0.94	0.68–1.29	1.19	0.84–1.69
ULBP2	221291_at	**1.52**	1.04–2.23	0.96	0.54–1.69	1.08	0.79–1.49	1.13	0.80–1.60
ULBP3	231748_at	1.16	0.73–1.85	1.08	0.54–2.16	0.74	0.46–1.20	**2.71**	1.41–5.21

Red, purple, and blue letters indicate inhibitory, inhibitory/stimulatory, and stimulatory pathways, respectively. Bold HR: statistically significant ($p < 0.05$) increase or decrease.

Table 2. Hazard ratio (HR) and 95% confidence interval (CI) for overall survival based on the expression levels of immune checkpoints in TNBC subtypes.

		BL1-TNBC		**BL2-TNBC**		**IM-TNBC**		**ME-TMBC**		**MSL-TMBC**		**LAR-TMBC**	
Gene	**ID**	**HR**	**95% CI**	**HR**	**95% CI**	**HR**	**95% CI**	**HR**	**95% CI**	**HR**	**95% CI**	**HR**	**95% CI**
CD274	227458_at 223834_at	**0.23**	0.07–0.69	0.44	0.13–1.47	0.78	0.24–2.56	0.64	0.29–1.37	0.5	0.17–1.50	1.24	0.53–2.88
PDCD1LG2	220049_s_at	0.57	0.25–1.30	0.59	0.22–1.63	**0.43**	0.18–1.02	0.75	0.39–1.45	1.19	0.43–3.32	0.81	0.44–1.47
NCAM1	209968_s_at 212843_at 214952_at 217359_s_at	1.08	0.50–2.33	1.85	0.67–5.10	1.13	0.50–2.55	0.92	0.48–1.78	0.66	0.23–1.86	0.93	0.51–1.69
CD276	224859_at 1552914_a_at	1.01	0.40–2.55	1.73	0.51–5.92	1.70	0.50–5.82	1.49	0.68–3.26	0.99	0.34–2.92	0.42	0.17–1.03
VTCN1	219768_at	1.17	0.54–2.54	0.62	0.22–1.71	**2.51**	1.10–5.73	1.14	0.59–2.19	1.03	0.37–2.86	0.89	0.49–1.62

CD200	209583_s_at	0.50	0.22–1.09	**3.17**	1.02–9.84	0.62	0.28–1.37	1.24	0.65–2.39	1.02	0.37–2.83	0.72	0.39–1.32
LGALS9	203236_s_at	0.88	0.41–1.90	**4.87**	1.52–15.6	1.14	0.51–2.52	0.83	0.43–1.60	**0.29**	0.09–0.92	0.83	0.45–1.51
HMGB1	200679_x_at 200680_x_at 214938_x_at 216508_x_at	1.20	0.55–2.60	0.57	0.21–1.58	0.79	0.36–1.75	0.79	0.41–1.52	1.33	0.48–3.67	1.25	0.69–2.29
CEACAM1	206576_s_at 209498_at 210610_at 211883_x_at 211889_x_at	1.32	0.61–2.85	1.09	0.40–2.92	1.14	0.52–2.51	1.03	0.53–1.98	0.60	0.21–1.70	0.83	0.46–1.52
ADORA2A	205013_s_at	0.60	0.27–1.32	0.97	0.36–2.61	0.78	0.36–1.73	1.59	0.82–3.09	0.51	0.18–1.43	0.87	0.48–1.59
HLA-G	210514_x_at 211528_x_at 211529_x_at 211530_x_at	1.22	0.56–2.65	0.42	0.15–1.21	0.56	0.25–1.25	0.99	0.52–1.90	0.55	0.19–1.54	0.67	0.36–1.22
SIRPA	202895_s_at 202896_s_at 202897_at 217024_x_at	1.21	0.55–2.64	1.22	0.45–3.28	1.33	0.59–2.97	0.87	0.45–1.67	0.68	0.24–1.92	0.89	0.49–1.63
THBS1	201107_s_at 201108_s_at 201109_s_at 201110_s_at 215775_at	0.88	0.40–1.91	1.94	0.70–5.35	1.50	0.67–3.33	0.78	0.41–1.50	2.76	0.94–8.12	1.03	0.57–1.88
CLEC2D	220132_s_at	0.73	0.34–1.60	1.53	0.56–4.15	1.16	0.53–2.55	0.62	0.32–1.21	0.56	0.20–1.58	0.68	0.37–1.26
CDH1	201130_s_at 201131_s_at 209414_at 209415_at 209416_s_at 211865_s_at	1.13	0.52–2.44	1.41	0.52–3.78	1.36	0.61–2.99	**1.96**	0.99–3.87	0.66	0.23–1.85	**0.33**	0.17–0.64
CDH2	203440_at 203441_s_at	0.83	0.39–1.81	1.30	0.48–3.54	1.05	0.48–2.30	1.30	0.67–2.51	0.75	0.27–2.08	1.27	0.70–2.32
CDH3	203256_at 206327_s_at 206328_at	1.34	0.61–2.92	1.72	0.62–4.76	1.76	0.76–4.10	1.49	0.77–2.90	1.54	0.55–4.33	0.74	0.40–1.35

HLA-E	200904_at 200905_x_at 217456_x_at	1.01	0.47–2.17	0.83	0.31–2.25	0.58	0.26–1.30	1.13	0.59–2.18	1.10	0.40–3.07	0.71	0.39–1.31
CD80	207176_s_at	0.69	0.32–1.50	1.28	0.48–3.43	0.95	0.43–2.08	0.59	0.30–1.15	0.37	0.12–1.08	**1.92**	1.03–3.58
CD86	205685_at 205686_s_at 210895_s_at	0.96	0.45–2.08	0.49	0.17–1.41	0.74	0.33–1.65	0.81	0.42–1.56	0.88	0.31–2.50	0.85	0.47–1.55
PVR	212662_at 214443_at 214444_s_at 216283_s_at 32699_s_at	0.87	0.40–1.88	**2.78**	0.96–8.04	1.08	0.49–2.41	0.61	0.31–1.19	1.24	0.45–3.44	1.44	0.78–2.64
PVRL2	203149_at	1.37	0.63–2.97	0.93	0.35–2.47	1.63	0.72–3.70	1.21	0.63–2.33	1.09	0.39–3.00	0.83	0.45–1.51
HHLA2	220812_s_at	1.01	0.47–2.19	1.14	0.53–3.80	1.54	0.69–3.44	1.03	0.54–1.99	0.48	0.16–1.41	**1.96**	1.05–3.65
HLA-A	213932_x_at 215313_x_at	0.98	0.45–2.12	0.70	0.26–1.94	0.46	0.21–1.05	1.07	0.56–2.06	0.53	0.19–1.49	1.15	0.63–2.09
HLA-B	208729_x_at 209140_x_at 211911_x_at	0.73	0.33–1.58	0.91	0.34–2.45	0.71	0.32–1.57	0.81	0.42–1.56	0.66	0.23–1.85	0.80	0.44–1.46
HLA-C	208812_x_at 211146_at 211799_x_at 214459_x_at 216526_x_at	0.94	0.43–2.02	0.84	0.31–2.25	0.70	0.32–1.54	0.78	0.41–1.51	0.52	0.19–1.47	0.98	0.54–1.79
TNFRSF14	209354_at	0.57	0.26–1.25	2.16	0.75–6.22	**0.39**	0.17–0.90	1.67	0.85–3.27	0.51	0.18–1.45	1.27	0.70–2.33
CD48	204118_at	**0.41**	0.18–0.94	0.35	0.11–1.09	0.99	0.45–2.17	**0.46**	0.23–0.92	0.41	0.14–1.22	0.88	0.48–1.60
ICOSLG	211197_s_at 211198_s_at 211199_s_at 213450_s_at	1.52	0.70–3.31	0.86	0.32–2.32	0.88	0.40–1.95	1.08	0.56–2.08	0.88	0.32–2.44	0.95	0.52–1.72
TNFSF4	207426_s_at	1.26	0.58–2.74	2.18	0.78–6.08	0.57	0.26–1.28	0.62	0.32–1.21	1.52	0.55–4.22	0.81	0.44–1.51
TNFSF7	206508_at	0.89	0.41–1.94	0.75	0.28–2.00	0.48	0.21–1.09	1.00	0.52–1.92	0.82	0.30–2.26	1.24	0.68–2.28
TNFSF8	207216_at	1.62	0.74–3.52	1.35	0.50–3.67	1.30	0.59–2.86	1.14	0.59–2.20	0.63	0.22–1.79	1.03	0.56–1.87
TNFSF9	206907_at	0.66	0.30–1.43	0.47	0.17–1.31	0.69	0.31–1.53	0.84	0.44–1.62	0.69	0.24–1.94	1.30	0.71–2.39
TNFSF10	202687_s_at 202688_at 214329_x_at	0.51	0.23–1.15	0.50	0.18–1.40	1.14	0.52–2.50	1.19	0.62–2.29	1.62	0.58–4.55	0.83	0.45–1.53
TNFSF14	207907_at	1.24	0.57–2.68	0.42	0.14–1.20	1.12	0.51–2.48	0.95	0.50–1.84	0.96	0.35–2.69	0.61	0.33–1.11
TNFSF15	221085_at	0.87	0.40–1.88	1.46	0.54–3.94	1.22	0.55–2.68	0.84	0.44–1.63	1.09	0.39–3.01	1.04	0.57–1.89

TNFSF18	221371_at	1.23	0.57–2.66	1.16	0.44–3.12	1.00	0.46–2.20	0.96	0.50–1.85	0.43	0.15–1.26	1.23	0.67–2.25
BAT3	201255_x_at 210208_x_at 213318_s_at	0.68	0.31–1.49	0.91	0.34–2.42	0.91	0.41–2.01	1.09	0.56–2.10	**0.30**	0.09–0.94	0.67	0.37–1.23
MICA	205904_at 205905_s_at	1.45	0.66–3.15	1.18	0.42–3.05	1.25	0.56–2.78	1.95	0.99–3.86	0.97	0.35–2.70	0.76	0.42–1.39
MICB	206247_at	**0.40**	0.17–0.92	1.11	0.41–2.96	0.49	0.22–1.10	0.57	0.29–1.11	0.48	0.17–1.37	0.86	0.47–1.57
ULBP1	221323_at	1.10	0.51–2.39	0.74	0.27–1.99	1.66	0.71–3.88	1.84	0.94–3.59	**0.32**	0.11–0.94	1.20	0.66–2.21
ULBP2	221291_at	1.43	0.66–3.12	**3.23**	1.04–10.0	1.66	0.75–3.71	1.46	0.75–2.83	0.95	0.34–2.63	0.77	0.42–1.41
ULBP3	231748_at	1.94	0.73–5.17	0.67	0.21–2.12	0.99	0.30–3.27	0.84	0.39–1.81	1.03	0.36–2.95	1.43	0.61–3.36

Red, purple, and blue letters indicate inhibitory, inhibitory/stimulatory, and stimulatory pathways, respectively. Bold HR: statistically significant ($p < 0.05$) increase or decrease. Basal-like 1 (BL1); basal-like 2 (BL2); immunomodulatory (IM); mesenchymal (ME); mesenchymal stem-like (MSL); luminal androgen receptor (LAR) TNBC subtypes.

4. Discussion

We have identified critical immune checkpoints for poor overall survival in breast cancer subtypes: ULBP2 in both basal-like breast cancer and basal-like 2 TNBC and VTCN1 in immunomodulatory TNBC. Although ULBP2 expression resulted in a longer relapse-free survival in breast cancer patients [19], it was negative to breast cancer patient survival, showing a negative correlation with CD8+ T cell [20]. ULBP2 was highly expressed in bone metastases of breast cancer than in primary tumors. ADAM17 facilitated the shedding of soluble ULBP2 from the surface of breast cancer cells, exhibiting resistance to killing by NK cells [21]. Down-regulation of ULBP2 suppressed MDA-MB-231 TNBC cell proliferation and migration [22]. These results support in part our finding that ULBP2 is associated with a poor overall survival in basal-like breast cancer and basal-like 2 TNBC subtypes.

VTCN1 was highly expressed in both breast cancer and stromal cells but was not associated with survival in breast cancer [23]. VTCN1 was also identified as a specific target for basal-like immunosuppressed TNBC, showing worst prognosis [24]. But VTCN1 has improved overall survival in luminal A subtype (Figure 4A), indicating a subtype-specific manner. Because VTCN1 levels are dominant in basal-like subtype compared to luminal A subtype (Figure 2), its high levels may negatively affect prognosis in basal-like subtype including TNBC. VTCN1 expression was progressively increased in breast cancer, showing a significant association between a high proportion of VTCN1 positive cells in invasive ductal carcinomas and decreased number of tumor-infiltrating lymphocytes [25]. Interestingly, the VTCN1 genetic variants might relate to the risk of breast cancer [26], appearing importance of genetic variants in immune checkpoints. VTCN1 contributed to dimethylbenz(a)anthracene-induced breast cancer progression, accompanied by CD8+ T cell exhaustion [27]. In human breast cancer, VTCN1 is associated with reduced CD8+ T cell infiltration [28]. Interestingly, VTCN1 was associated with survival benefit for patients with metastatic TNBC treated with carboplatin plus anti-PD-L1 but it does not affect the survival of patients with early breast cancer receiving chemotherapy plus anti-PD-1 [29]. EMT6 mouse mammary tumors with cell-surface VTCN1 expression were more resistant to immunotherapy [29]. VTCN1-targeted antibody-drug conjugates induced complete tumor regressions in xenograft models of breast cancer as well as in a syngeneic breast cancer model that is refractory to PD-1 [30]. Silencing VTCN1 decreased cell viability of MCF-7 and T47D breast cancer cells [31], indicating that VTCN1 may serve as a potential target for breast cancer, warranting further study to identify VTCN1 driven subtypes.

Expression of CD274 is rare in breast cancer, but markedly enriched in basal-like subtype [32], and is higher in HER2-positive and TNBC [33], supporting our results that exhibit high levels of CD274 in HER2 and basal-like subtypes. Increased CD274 expression was significantly associated with a good overall survival in breast cancer [34,35], improving overall survival in basal-like subtype [36]. These studies are similar to our results showing CD274-induced improved overall survival in basal-like subtype and basal-like 1 TNBC (Figure 4A,B).

HLA-A, HLA-B, and HLA-C were associated with favorable overall survival in basal-like subtype and metastatic breast cancer [37–39], in line with our results (Figure 4A). On the other hand, HLA-A had no association with survival in TNBC and HER2 subtype [40], as indicated by no change in HER2 and TNBC subtypes (Tables 1 and 2). HLA-G showed controversial results in breast cancer progression. Levels of soluble HLA-G were higher in breast cancer patients compared to healthy women [41], indicating a potential target of HLA-G. HLA-G expression was inversely associated with the density of tumor infiltrating lymphocytes (TIL), indicating that HLA-G is a negative regulator of TIL. Accordingly, patients with high HLA-G/low TIL status had a higher risk of recurrence than those with low HLA-G/high TIL status [42]. However, HLA-G expression did not result in poor clinical outcome of cancer patients, implying not required for an inhibited tumor-immune response and tumor progression [43]. The expression of HLA-G was higher in patients with shorter survival time [44]. HLA-G 3′UTR variant might reduce overall survival in locally advanced, non-metastatic breast cancer patients [45]. On the other hand, HLA-G was associated with improved overall survival in basal-like subtype [38], which was similar with our results (Figure 4A). Although HLA-E had improved overall survival in basal-like subtype (Figure 4A), it resulted in worse overall survival in breast cancer [46]. Inconsistent results of HLA-E on survival remain to be determined.

CLEC2D, TNFRSF14, TNFSF14, TNFSF8, MICB, and BAT3 show an improved overall survival (Figure 4A,B). However, the data on the relationship between these genes and breast cancer survival are limited at this point. HHLA2 showed a poor overall survival in luminal androgen receptor TNBC (Figure 4B), requiring further study because of the limited data available on the roles of HHLA2 in breast cancer survival.

CD86 had a favorable overall survival in basal-like subtype [47], in line with our results (Figure 4A). Although HER2 subtype shows a CD86-induced longer overall survival (Figure 4A), other report shows that CD86 is not associated with overall survival [47], requiring further investigation. Also, CD80 had a good overall survival

in basal-like subtype [47] as also shown in our results (Figure 4A), but shows a poor overall survival in luminal androgen receptor TNBC (Figure 4B), requiring further study on breast cancer subtype-specific manner. CD48 was associated with a good overall survival of breast cancer patients [48], being consistent with our results (Figure 4A,B).

Interestingly, luminal B subtype shows a poor overall survival with high levels of THBS1, CDH2, and ULBP3 (Figure 4A). THBS1 was not associated with overall survival in breast cancer [49] but it was associated with CNS metastases in TNBC tumors [50] and shorter overall survival in advanced breast cancer patients [51]. Up-regulation of THBS1 was associated with chemotherapy resistance in breast cancer patients [52]. High plasma levels of THBS1 were associated with an increased occurrence of brain metastasis in HER2-enriched patients [53]. These results indicate the important roles of THBS1 in advanced breast cancer. CDH2 had poor overall survival in breast cancer patients [54,55], supporting in part our results in luminal B subtype (Figure 4A). Although ULBP3 showed no associations with recurrence-free survival in breast cancer patients [19], its poor overall survival in luminal B subtype (Figure 4A) reminds to be determined.

Particularly, basal-like 2 TNBC showed poor overall survival with high levels of CD200 and PVR (Figure 4B). CD200 had no associations with overall survival in breast cancer patients [47]. Because CD200 is closely associated with a basal/stem and invasiveness gene signature, which represents breast cancer stem cells [56], its poor overall survival in basal-like 2 TNBC remains to be determined. PVR expression is associated with more aggressive breast cancer, such as HER2 subtype and TNBC [57]. High expression of PVR is an independent prognostic marker with a poor outcome for breast cancer patients [57], in line with our results in basal-like 2 TNBC. Also, no change in overall survival was reported in TNBC patients with high expression of PVR [58]. LGALS9 gene is controversial, showing a poor overall survival in basal-like 2 TNBC and a good overall survival in mesenchymal stem-like TNBC (Figure 4B), requiring further study to clarify LGALS9-induced overall survival in breast cancer subtypes. Increased PDCD1LG2 expression was associated with a good overall survival in breast cancer [34,35], supporting in part PDCD1LG2-related good overall survival in immunomodulatory TNBC (Figure 4B).

CDH1 is also inconsistent with a poor overall survival in mesenchymal TNBC and a good overall survival in luminal androgen receptor TNBC (Figure 4B). Deregulation of CDH1 plays a crucial role in breast cancer metastases with worse prognosis and shorter overall survival [59]. CDH1 truncating mutation was related to poor survival in patients with breast invasive lobular carcinoma [60]. CDH1 and its promoter methylation showed a poor overall survival in breast cancer [55,61,62] but CDH1-induced overall survival is still inconsistent among some studies, showing poor, good, and no association [63]. Although ULBP1 showed an improved overall survival in mesenchymal stem-like TNBC (Figure 4B), it was associated with a poor overall survival in patients with BRCA [64], remaining to be determined.

Basal-like subtype had higher levels of NCAM1, SIRPA, and CDH3 (Figure 2), which did not associate with overall survival. However, NCAM1 was identified as a potential therapeutic target and biomarker for breast cancer [65]. Although high expression of NCAM1 was correlated with poor prognosis in luminal A subtype samples from TCGA [66], NCAM1 in this study did not affect overall survival in luminal A subtype. The poor prognosis of breast cancer patients with high expression of CD47 might be due to an active CD47/SIRPA signaling in circulating cells [67]. Therefore, basal-like subtype with high levels of SIRPA may have a worse survival rate in response to CD47. CDH3 often results in increased invasiveness of tumor cells [68] and mediates stem cell properties [69], reflecting aggressiveness in basal-like subtype with high levels of CDH3 compared to other subtypes (Figure 2). Because CDH3 has been reported to have a poor overall survival in breast cancer [55], the differential results remain to be determined. Both basal-like and HER2 subtypes showed high levels of ICOSLG and TNFSF9 (Figure 2), but these immune checkpoints had no association with overall survival. Although trastuzumab is a targeted therapy for HER2 subtype, ICOSLG is identified as a potential biomarker of trastuzumab resistance [70], likely decreasing therapeutic effectiveness.

In conclusion, ULBP2 in both basal-like subtype and basal-like 2 TNBC and VTCN1 in immunomodulatory TNBC may be a core molecular target for immune checkpoints in breast cancer subtypes, based on signatures for immune checkpoints and overall survival. Various immune checkpoints may differentially influence overall survival in breast cancer subtypes because of tumor heterogeneity, which causes inconsistent results of some immune checkpoints. Although specific immune checkpoints are predominant in some breast cancer subtypes, the final net balance of total immune checkpoints may affect critically overall survival in breast cancer by exerting inhibitory or stimulatory interaction with immune cells. The immune checkpoints identified in this study could potentially serve as actionable drug targets for treating TNBC, a subtype with limited therapeutic options due to the absence of specific targets and poorer prognosis, ultimately contributing to enhanced overall survival.

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