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ORIGINAL ARTICLE

Gene expression profile of peripheral blood mononuclear cells may contribute to the identification and immunological classification of breast cancer patients

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Abstract

Background It has been reported that the gene expression profile of peripheral blood mononuclear cells (PBMCs) exhibits a unique gene expression signature in several types of cancer. In this study, we aimed to explore the breast cancer patient-specific gene expression profile of PBMCs and discuss immunological insight on host antitumor immune responses.

Methods We comprehensively analyzed the gene expression of PBMCs by RNA sequencing in the breast cancer patients as compared to that of healthy volunteers (HVs). Pathway enrichment analysis was performed on MetaCore^{tm} to search the molecular pathways associated with the gene expression profile of PBMCs in cancer patients compared with HVs.

Results We found a significant unique gene expression signature, such as the Toll-like receptor (TLR) 3- and TLR4-induced Toll/interleukin-1 receptor domain-containing adapter molecule 1 (TICAM1)-specific signaling pathway in the breast cancer patients as compared to that of healthy volunteers. Distinctive immunological gene expression profiles also showed the possibility of classifying breast cancer patients into subgroups such as T-cell inhibitory and monocyte-activating groups independent of known phenotypes of breast cancer.

Conclusions These preliminary findings suggest that evaluation of gene expression patterns of PBMCs might be both a less invasive diagnostic procedure and a useful way to reveal immunological insight of breast cancer, including biomarkers for cancer immunotherapy, such as immune checkpoint inhibitor therapy.

Keywords Immuno-oncology · Gene expression profile · RNA sequencing · Peripheral blood mononuclear cells · Immune checkpoint molecule

Introduction

In the interaction between cancer cells and immune cells, the presence of cancer cells causes immune cells to undergo various phenotypic and functional changes and the affected immune cells kill cancer cells or promote proliferation and metastasis of cancer cells [1–3]. After such changes occur, it is expected that the systemic reactivity of immune cells to cancer cells will be different in individual patients. This might depend on both the characteristics of the cancer cells and the characteristics of the immune cells of the host. Based on these concepts, attempts have been made to detect the presence of cancer cells by analyzing the gene expression profile of peripheral blood mononuclear cells (PBMCs) in patients with lung cancer and pancreatic cancer and the significant gene expression profile of PBMCs in the cancer patients was detected [4, 5]. These findings suggested

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that analysis of peripheral immune cells might be appropriate to evaluate host immune reaction against cancer cells in addition to analyzing tumor-infiltrated immune cells. If we are able to analyze immune reaction related to cancer progression or efficacy of immune-related cancer therapy by evaluating PBMCs, it will be clinically useful because blood sample collection is more patient friendly as compared to the tumor tissue sampling.

As an exploratory study for future clinical applications of immunological evaluation of efficacy for cancer immune therapeutics by liquid biopsy, in this study, we comprehensively analyzed gene expression in PBMCs from breast cancer (BC) patients and healthy volunteers (HVs) by RNA sequencing (RNA-seq) to identify specific gene expression patterns that can potentially distinguish between these two groups. We also considered that classification of BC patients using breast cancer-specific genes in PBMCs could lead to new immunologic insights in BC patients, reflecting interaction between biological characteristics of host antitumor immunity and cancer immune therapeutics. Thus, we also tried to identify the distinctive BC subgroups that can be distinguished by gene expression patterns of PBMCs in BC patients.

Table 1 Demographics of BC patients and HVs in the study

Characteristic	PBC	MBC	HV
Number	6	7	3
Female	6 (100%)	7 (100%)	3 (100%)
Age (mean ± SD)	56.2 ± 13.1	63.5 ± 9.1	31.3 ± 1.1
Stage			
I	3		NA
II	3		NA
III			NA
IV		7	NA
Phenotype			
Luminal	2	4[a]	NA
HER2	2	3[a]	NA
TNBC	2	1	NA
Ki67 (mean ± SD)	37.9 ± 29.9	26.1 ± 26.6	NA
Therapeutic status	None	Endocrine therapy (1) Chemotherapy (3) None (3)	None

BC breast cancer, *PBC* primary breast cancer, *MBC* metastatic breast cancer, *HV* healthy volunteer, *TNBC* triple-negative breast cancer

[a]Includes one patient with luminal + HER2 phenotype

Materials and methods

Study population

The study protocol was approved by the Ethics Committee for Clinical Research, Kyoto University Hospital (authorization number G424). Written informed consent was obtained from all patients and HVs before enrollment into the study. For this study, 13 BC patients (6 with primary BC and 7 with metastatic breast cancer) and 3 HVs were recruited. Baseline demographic information for all groups is detailed in Table 1.

Isolation of total RNA from PBMCs

PBMCs were isolated from whole blood using BD Vacutainer CPT Cell Preparation Tubes (BD, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and immediately frozen in liquid nitrogen and stored at − 80 °C.

RNA-seq

RNA-seq was performed using an Ion Proton System for next-generation sequencing (Life Technologies) at the Medical Research Support Center, Kyoto University. Briefly, for each of the samples, 10 ng of total RNA was reverse transcribed using the Ion AmpliSeq Transcriptome Human Gene Expression kit following the manufacturer's protocol (Life Technologies). The expression level of genes was normalized as reads per million mapped reads (RPM).

Statistical analysis

Difference in gene expression level of BC patients and HVs were evaluated by *t* test and false discovery rate (FDR). Genes with a fold change in expression level of < 0.5 or > 2.0 and FDR-corrected P values < 0.05 in comparison between BC patients and HVs were defined as significantly differentially expressed genes (SDEGs) between BC patients and HVs. Log2 transformation was applied to all fold change ratio of SDEGs in hierarchical cluster analysis. Cluster analysis was performed by Pearson correlation using Mev TM4 [6].

Molecular pathway enrichment analysis

Using SDEGs, pathway enrichment analysis was performed on MetaCore[tm] (Thomson Reuters, New York, NY, USA) to search the molecular pathways associated with the gene expression profile of PBMCs in cancer patients compared with HVs. The pathways with P values less than 0.05 were selected as significant pathways.

Results

Profile of significantly differentially expressed genes (SDEGs)

The number of genes analyzed in RNA-seq was 18,059. Of these, 4092 genes and 1442 genes were found to be significantly different between BC patients and healthy volunteers (HVs) at $P < 0.05$ and false discovery rate (FDR) < 0.05, respectively. Of these, 353 genes were two-fold upregulated (Supplementary Table 1) and 36 genes were 0.5-fold downregulated (Supplementary Table 2) in PBMCs of BC patients.

Hierarchical cluster analysis

Using SDEGs, hierarchical clustering of RNA-seq data clearly identified two distinct clusters, a BC group and an HV group (Fig. 1a), and the BC patient group was further divided into 2 distinct groups (Fig. 1b). Characteristics of individual samples are shown in Table 2.

A TLR3- and TLR4-induced TICAM1-specific signaling pathway and a Th17, Th22, and Th9 cell differentiation pathway are associated with the BC patient group

To explore the immunological significance of gene expression profiling of PBMCs in BC patients, molecular pathways that were possibly associated with BC patients were

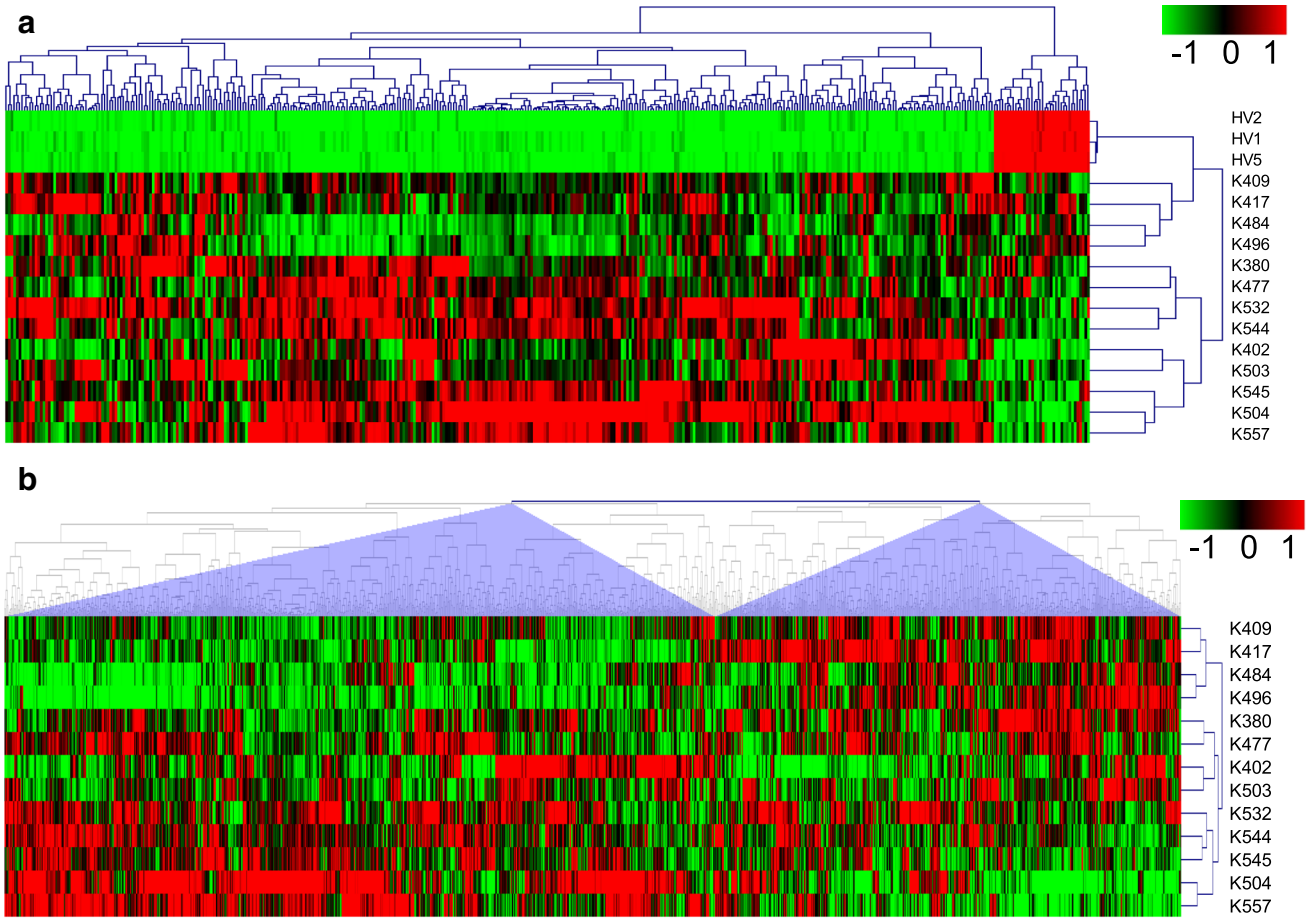

Fig. 1 **a** Heatmap for breast cancer and healthy control data. A total of 407 genes showing both FDR-corrected $P < 0.05$ (Student's t test, two-tailed, heteroscedastic) and large difference (fold change < 0.5 or > 2.0) between healthy controls and breast cancer patients were used for the heatmap. Log2-transformed intensities were normalized to Z scores for each gene. Pearson correlation was used for clustering genes. **b** Heatmap for only breast cancer data. A total of 1441 genes showing FDR-corrected $P < 0.05$ (Student's t test, two-tailed, heteroscedastic) between healthy controls and breast cancer patients were used for the heatmap. Log2-transformed intensities were normalized to Z scores for each gene. Pearson correlation was used for clustering genes

Table 2 Individual sample characteristics

Sample	Age	Ki67	ER	HER2	Disease status
496-1	66	20	+	+	MBC
402	69	15	−	−	MBC
417	74	49	−	+	PBC
484	58	24	−	+	PBC
504	44	60.3	−	−	PBC
503	49	4	+	−	PBC
477	51	10.5	+	−	PBC
380	66	79.8	−	−	MBC
545	61	10.8	+	+	MBC
409	78	10	−	+	MBC
557	51	21.1	+	−	MBC
544	54	NA	+	−	MBC
HV1	32				
HV2	32				
HV5	30				
532		NA	−	−	PBC

PBC primary breast cancer, *MBC* metastatic breast cancer, *HV* healthy volunteer

evaluated by MetaCore. Fifty pathways were identified to be associated with BC patients, and of these, the top 2 immune response-related pathways were a TLR3- and TLR4-induced Toll-like receptor adaptor molecule 1-specific (TICAM1-specific) signaling pathway and a Th17, Th22, and Th9 cell differentiation pathway (Table 3). TLR3, TLR4, TNF receptor-associated factor 6 (TRAF6), and receptor-interacting protein kinase 1 (RIPK1) were found to be associated with the TLR3- and TLR4-induced TICAM1-specific signaling pathway, and CD86 and TGF-beta receptor type I were associated with the Th17, Th22, and Th9 cell differentiation pathway, and all these genes were upregulated in BC patients compared with HVs.

BCR and CRTH2 signaling pathways in Th2 cells may be associated with characterization of two subsets of BC patients

We determined that BC patients are divided into two distinct subsets by cluster analysis (Fig. 1b). To understand the immunological characteristics of those 2 subsets, pathway analysis was performed to determine the genes that could be associated with each subset. SDEGs between the two BC patient groups showed a trend correlation between B-cell receptor (BCR) immunological pathways and chemoattractant receptor-homologous molecule on Th2 (CRTH2) signaling in Th2 cells in 2 BC patient subsets. Genes found to be associated with the BCR pathway are Bcl-XL, EGR1, p70 S6 kinase 1, Bcl-10, calcineurin A (catalytic), SOS, calmodulin, SHIP, PI3K reg class IA, IKK-alpha, and TAK1 (MAP3K7), and those associated with CRTH2 signaling are Bcl-XL, calcineurin A (catalytic), calmodulin, PKC, Apaf-1, and G-protein (Table 4).

Table 3 Pathway enrichment analysis on all samples including BC patients and HVs

No.	Maps	FDR	Mapped/total genes	Network objects from active data
1	Immune response_TLR3 and TLR4-induced TICAM1-specific signaling pathway	8.371E−02	4/20	TLR3, TLR4, TRAF6, RIPK1
2	Putative pathways for stimulation of fat cell differentiation by bisphenol A	6.635E−01	2/32	PI3K cat class IA, C/EBPbeta
3	Development_EGFR signaling pathway	6.302E−01	4/71	PI3K cat class IA, SOS, c-Myc, IKK-beta
4	Immune response_Th17, Th22 and Th9 cell differentiation	7.362E−01	2/39	CD86, TGF-beta receptor type I
5	Immune response_CD40 signaling	3.033E−02	9/65	STAT5A, PI3K cat class IA, TRAF3, p27KIP1, TRAF5, CD86, TRAF6, IKK-beta, FasR (CD95)
6	Riboflavin metabolism	7.015E−01	1/14	PPAL
7	Ca(2+)-dependent NF-AT signaling in cardiac hypertrophy	8.634E−01	2/57	PKA-reg (cAMP-dependent), G-protein alpha-i family
8	Muscle contraction_S1P2 receptor-mediated smooth muscle contraction	6.379E−01	2/30	G-protein alpha-12, MRLC
9	Immune response_IL-12 signaling pathway	3.809E−02	5/23	STAT5A, STAT5, G6NT, IL-12RB1, IFN-gamma
10	Cytoskeleton remodeling_Keratin filaments	7.004E−01	2/36	Tubulin alpha, tubulin gamma 1

Lists of pathways ranked by enrichment analysis. In total, 18,057 genes showing FDR-corrected $P < 0.05$ (Student's t test, two-tailed, heteroscedastic) between HVs and BC patients were used for this analysis. Enrichment analysis with both signal and intersection distribution were conducted and pathways were ranked based on the differentially affected

BC breast cancer, *HV* healthy volunteer, *FDR* false discovery rate

Table 4 Pathway enrichment analysis of BC patients

No.	Maps	FDR	Mapped/total genes	Network objects from active data
1	Apoptosis and survival_Role of PKR in stress-induced apoptosis	3.158E−03	12/53	PP2A regulatory, Caspase-7, PP2A catalytic, eIF2S1, IRF1, PKR, TNF-R1, Caspase-8, IKK-alpha, TARBP2, TAK1(MAP3K7), eIF4E
2	Transport_Clathrin-coated vesicle cycle	5.172E−03	13/71	TIP47, PI3K reg class III (p150), Syntaxin 7, GS15, GCC2, RAB9P40, EEA1, VAMP4, ARF1, Epsin 1, Rabaptin-5, Rab-9, VPS45A
3	Translation_Regulation of EIF4F activity	5.172E−03	11/53	EGF, p70 S6 kinase1, SOS, PP2A catalytic, RHEB2, eIF4G1/3, PI3K reg class IA, MEKK1(MAP3K1), eIF4G2, TAK1(MAP3K7), eIF4E
4	Transcription_Sirtuin6 regulation and functions	5.172E−03	12/64	SCAP, SREBP1 precursor, S2P, c-IAP2, Sirtuin1, SREBP1 (nuclear), Histone H3, LKB1, SOD2, SREBP1 (Golgi membrane), HMDH, FOXO3A
5	Development_Growth factors in regulation of oligodendrocyte precursor cell survival	5.172E−03	9/37	PDGF-A, Bcl-XL, 14-3-3 beta/alpha, PI3K reg class IA (p85), Fyn, p70 S6 kinase1, PI3K reg class IA, IKK-alpha, p90Rsk
6	Transport_Rab-9 regulation pathway	5.172E−03	5/10	TIP47, GCC2, RAB9P40, IGF-2 receptor, Rab-9
7	Development_PIP3 signaling in cardiac myocytes	5.172E−03	10/47	Bcl-XL, G-protein alpha-12 family, p70 S6 kinase1, SOS, RHEB2, PI3K reg class IA, PARD6, p90Rsk, FOXO3A, 14-3-3
8	Cytoskeleton remodeling_Reverse signaling by ephrin B	5.902E−03	8/31	Tubulin alpha, SOS, PINCH, RGS3, G-protein alpha-i family, SDF-1, FAK1, Tubulin (in microtubules)
9	Proteolysis_Role of Parkin in the Ubiquitin–Proteasomal Pathway	5.902E−03	7/24	Tubulin beta, Alpha-synuclein, Tubulin alpha, SIAH2, HSP70, Caspase-8, FBXW7
10	Development_Thromboxane A2 signaling pathway	5.902E−03	10/50	EGF, PI3K reg class IA (p85), G-protein alpha-12 family, p70 S6 kinase1, PKC, PI3K reg class IA, TBXA2R, G-protein alpha-i family, Adenylate cyclase, G-protein alpha-13
11	IGF family signaling in colorectal cancer	5.902E−03	11/50	GIPC, Bcl-XL, ZNF143, p70 S6 kinase1, PI3K reg class IA (p85-alpha), SOS, IGF-2 receptor, IKK-alpha, MAT2A, eIF4E, Clusterin
12	Apoptosis and survival_BAD phosphorylation	6.326E−03	9/42	Bcl-XL, p70 S6 kinase1, Calcineurin A (catalytic), SOS, PP2C, PP2A catalytic, PI3K reg class IA, p90Rsk, 14-3-3
13	Development_Negative regulation of STK3/4 (Hippo) pathway and positive regulation of YAP/TAZ function	6.326E−03	11/62	EGF, TAZ, G-protein alpha-12 family, PAR1, Schwannomin (NF2), G-protein alpha-q/11, FRMD4A, SIAH2, HIPK2, STK3, LARG
14	Immune response_BCR pathway	6.326E−03	11/62	Bcl-XL, EGR1, p70 S6 kinase1, Bcl-10, Calcineurin A (catalytic), SOS, Calmodulin, SHIP, PI3K reg class IA, IKK-alpha, TAK1(MAP3K7)
15	Regulation of metabolism_Role of Adiponectin in regulation of metabolism	6.650E−03	9/43	SREBP1 precursor, TFAM, Sirtuin1, p70 S6 kinase1, ACADM, RHEB2, LKB1, SOD2, AdipoR1
16	Immune response_CRTH2 signaling in Th2 cells	7.522E−03	9/44	Bcl-XL, Calcineurin A (catalytic), Calmodulin, PKC, Apaf-1, G-protein alpha-i family, Adenylate cyclase, IL-13, 14-3-3

Lists of pathways ranked by enrichment analysis. In total, 1390 genes showing $P < 0.05$ (Mann–Whitney test) between M and K groups were used for this analysis

BC breast cancer, *FDR* false discovery rate

Both HVs and BC patients could be divided into immunological subgroups by known immune-activating and -inhibitory gene expression patterns

Although pathway analysis revealed that PBMCs of BC patients have a background in which innate and adaptive immunity is activated, it is well-known that when antitumor immune responses are activated, the immune checkpoint

factor is reinforced against the activation signal. Thus, to understand the immunological background in both HVs and BC patients, we specifically tested the expression level of immune activation-related genes such as CD109, NKG2D, CXCR4, CCR6, CD8A, KLRK1, CD248, CD4, neuropilin 1 (NRP1), Siglec14, CD14, CD80, and CD40, and immune checkpoint-related genes such as PD-1 (PDCD1), PD-L1 (CD274), CTLA-4, LAG3, TIM3 (HAVCR2), IDO1/2, and FOXP3. Individuals may be divided into 4 subgroups such as lymphocyte retention (CD8A, CD4, CD248, IDO1, and IDO2) (all HVs), T-cell inhibitory (PD-L1, PD1, CTLA4, FOXP3, and CCR3), monocyte activating (CD14, CD40, CD80, Siglec14, NRP1, and TIM3), and unknown (Fig. 2).

Discussion

In this study, we found that BC patients can be distinguished from HVs by gene expression profiling of PBMCs (Fig. 1a), suggesting that even peripheral immune cells show certain immunological responses to the presence of cancer cells. On the other hand, we acknowledge the limitations in this study. Especially, it is difficult to analyze the interaction between disease stage, subtype of tumor, cancer therapeutic efficacy, prognosis and the gene expression profile of PBMCs due to the small sample size in the study settings. Thus, we focused on interpreting the immunological properties in PBMCs specific to BC patients. Another limitation of this study might be that age of HVs un-matched that of BC patients. In fact, several studies demonstrated aging-related changes in the functions of innate immune cells, B-cells and T-cells [7–9].

As indicated by pathway analysis, the significant immunological changes in PBMCs in BC patients might be the activation of the TLR3 and TLR4 pathway, the activation of the Th17, Th22 and Th9 pathway, and the activation of the CD40 pathway (Table 3). TLR3 activates an innate immune response by RNA as a ligand, such as ssRNA and dsRNA [10, 11]; therefore, RNA encapsulated in exosomes secreted from cancer cells can elicit immune activity mediated by TLR3. On the other hand, TLR4 is mainly activated by LPS, though recent reports indicate that oxidized free fatty acid elicits an immune response via TLR4 [12]. Cancer

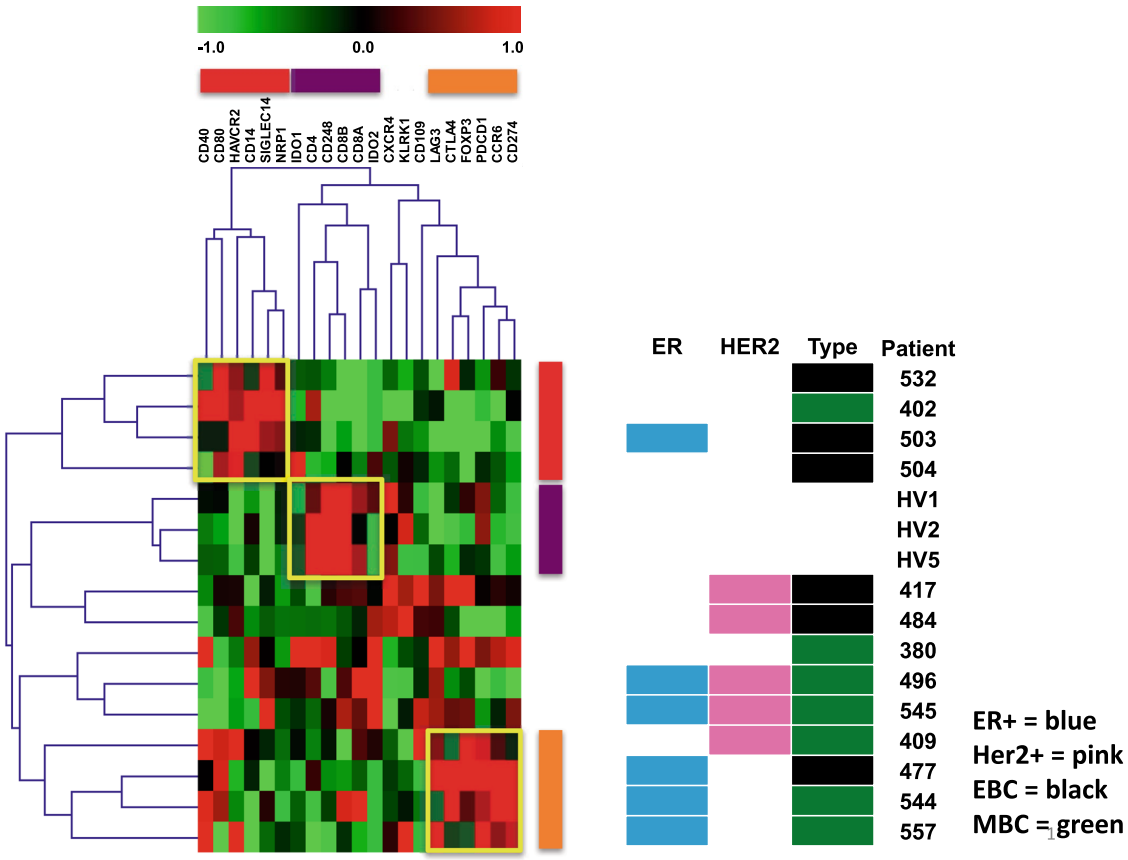

Fig. 2 Heatmap for known 21 immunological active and inhibitory genes. Log2-transformed intensities were normalized to *Z* scores for each gene. Pearson correlation was used for clustering genes. Colored bars indicate each immunological cluster. Red: monocyte-activating cluster (NRP-1, Siglec 14, CD14, TIM3, CD80 and CD40). Purple: lymphocyte-retention cluster (CD8A, CD56, CD248 and CD4). Orange: T-cell inhibitory cluster (CD274, CCR6, PDCD1, FOXP3, CTLA4 and LAG3)

cells produce a secretory form of phospholipase A2, which releases oxidized fatty acid into the circulation [13]. We postulated that TLR4 is specifically activated by oxidized free fatty acids present in the blood that have been released by the cancer cells' secreted phospholipase A2. Our data suggest that activation of the Th17 cell pathway is enhanced in the BC patients in this study (Table 3). Since Th17 cell is a T-cell subset related to the production of IL17 [14], IL17 production in peripheral blood may be promoted as the tumors are established. It has been reported that the expression level of IL17 in BC tissue correlates with its poor prognosis and chemoresistance [15, 16]. However, to the best of our knowledge, there has been no report on the correlation between the expression level of IL17 in peripheral blood and BC progression or certain treatment responses. Future study for analyzing the correlation between IL17 expression in blood and BC clinical status will be important.

Is it possible to classify cancer patients into specific groups according to immunological properties? Although statistically significant factors could not be found in this regard, the BCR pathway and CRTH2 signaling in Th2 cells were confirmed (Table 4). Cellular immunity activated via Th1 cells in BC patients with active Th2 cell signals might be attenuated [17]. One reason for such a condition might be the enhancement of an immune checkpoint factor such as PD1 or CTLA4 [18–21]. We also observed the possibility of classifying individuals including HVs and BC patients by cluster analysis of known immune-activating and -inhibitory gene expression patterns (Fig. 2). In this setting, while HVs may be classified as a lymphocyte function retention group, and BC patients might be classified as a T-cell inhibitory and monocyte-activating gene signature group, regardless of conventional tumor phenotypes such as luminal, HER2, and triple negative. Because we assumed that the treatment of chemotherapy or endocrine therapy for breast cancer patients could affect host immune cell functions, we performed additional analysis of gene expression pattern of known 21 immune-activating and -inhibitory genes on HVs and BC patients excluding 4 patients who had received chemotherapy or endocrine therapy (K409, K380, K496, K545) to eliminate the possible pretreatment effects on gene expression of immune cells. We found similar gene expression patterns to that of Fig. 2. There were minimum differences as compared to the full patients' analysis that IDO1 was out from a lymphocyte function retention group, HAVCR2 was out from the monocyte-activating gene signature group, LAG3 was out from a T-cell inhibitory gene signature group and CD8B entered into a lymphocyte function retention group. On the other hand, the member of patients in each cluster was same as the former analysis setting (Supplementary Figure 1). Because T-cell inhibitory gene signature includes upregulated expression of LAG3, CTLA4, FOXP3 and PDCD1 (PD-1) known as therapeutic target genes for current immune checkpoint inhibition therapy, it is presumed that immune checkpoint inhibition therapy might be effective in patients with a T-cell inhibitory gene signature in the gene expression profile test for PBMCs. It will be important to explore the significance of gene expression profile analysis of PBMCs as a biomarker for immune checkpoint inhibition therapy in the future.

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Compliance with ethical standards

Conflict of interest All authors declare no conflicts of interests in the authorship or publication of this article.

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