Clinical Relevance of Estrogen Reactivity in the Breast Cancer Microenvironment

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Research article

Keywords: breast cancer, estrogen reactivity, cancer genomics, tumor immune microenvironment, bioinformatics

Posted Date: July 7th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-671517/v1>

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Abstract

Background: Estrogen signals play an important role in the phenotype of estrogen receptor positive breast cancer (BC). However, comprehensive analyses of the effect of estrogen signals on the tumor microenvironment (TME) and treatment response in large cohorts of primary BC patients have been lacking. We aimed to test the hypothesis that estrogen reactivity effects gene expression and immune cell infiltration profiles in the TME and relates to response to chemotherapy (CT) and endocrine therapy (ET).

Methods: A total of 3091 BC cases were analyzed; 1075 from TCGA cohort, 1904 from METABRIC cohort, and 112 from Hokkaido University hospital cohort. We divided the group into estrogen reactivity-high and estrogen reactivity-low groups utilizing estrogen response genes.

Results: BC with high estrogen reactivity was related to Myc targets, Metabolism-related signaling, cell stress response, TGF-beta signaling, androgen response, and MTORC1 signaling gene sets in the TME. Low estrogen reactivity was related to immune-related proteins, IL2-STAT5 signaling, IL6-JAK-STAT3 signaling, KRAS signaling, cell cycle related gene sets, and EMT. In addition, BC with high levels of estrogen reactivity had low immune cytolytic activity, low levels of immunostimulatory cells, and high levels of immunosuppressive cells. It also had low levels of stimulatory and inhibitory factors of the cancer immunity cycle (CIC). Patients with high estrogen reactivity were also associated with a better prognosis. Regarding the effect of estrogen reactivity on treatment, patients who were treated with ET and CT but relapsed (BC with CT rec) were related with higher levels of E2F targets and G2M checkpoint, but lower levels of immunosuppressive M2 macrophages or Tregs cells. In addition, the TME in CT rec had higher levels of CIC regulators.

Conclusions: We demonstrated the relationship between estrogen reactivity and the profiles of immune cells and gene expression within the TME, as well as the treatment effect of CT given in addition to ET.

Background

Breast cancer (BC) has different subtypes of which approximately 75 percent are sensitive to the female sex hormone, estrogen [1]. These type of BCs, known as estrogen receptor positive (ER+) BC, have receptor sites that bind to estrogen which promotes cell growth and cancer progression.

Estradiol (17 β -estra-1,3,5 (10)-triene-3,17-diol) (E2) is a typical steroid hormone in estrogens, which is produced by theca cells and granulosa cells in the ovaries. Epidemiologic and experimental data implicate E2 as an important factor contributing to BC carcinogenesis [2]. E2 actions are mediated by genomic and non-genomic pathways. Genomic E2 signaling is primarily mediated by two isoforms of the receptor, ER α and ER β , both of which are nuclear transcription factors that bind to their specific ligand and several estrogens. E2 via ER α signaling stimulates cell proliferation, while ER β activation inhibits cell proliferation and promotes apoptosis. Non-genomic E2 signaling, known as ligand-independent ER α signaling, is primarily mediated by a membrane-anchored receptor called G protein-coupled estrogen receptor 1, in which target gene transcription occurs through second messengers and several transcription factors [3–5]. The cellular response to estrogens, estrogen reactivity, plays an important role in the molecular and genomic pathways of the hormone-responsive BC phenotype by affecting the transcriptional modulation of various genes such as proliferation regulators, growth factors, cell cycle, and apoptosis modulators [3]. Thus, it has been deeply studied to try to characterize

the structure of the process, and many advancements have been made [3]. We have demonstrated that low expressions of estrogen responsive genes were deeply involved in the late recurrence of BC patients [6]. On the other hand, the physical behavior of the extracellular matrix, stromal cells, fibroblasts, adipocytes, immune cells, and macrophages are known to modulate tumor cells and the tumor microenvironment (TME) to promote cancer survival and progression [7]. In addition, researchers have focused not only on cancer cells themselves, but also on TMEs as targets for cancer therapy [7]. Therefore, it is of interest to investigate estrogen reactivity in the TME as well as in breast tumors.

However, comprehensive analyses of how estrogen signals affect the components of the TME, such as immune cells and gene expression profiles, and how they affect treatment response in large cohorts of primary BC patients have been lacking. Gene Set Variant Analysis (GSVA) is the analysis to further explore the biological activity of a signaling pathway, which assesses the impact of specific pathways on the bioactivity of a set of genes of interest [8]. Utilizing ESTROGEN RESPONSE scores in GSVA, we can line up the patients in the order of how much estrogen reactivity is upregulated.

We aimed to test the hypothesis that estrogen reactivity affects gene expression and immune cell infiltration profiles in the TME, and that it relates to the response to chemotherapy (CT) given in addition to endocrine therapy (ET), utilizing our own institutional data and collected data from Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) and the cancer genome atlas (TCGA) BC cohorts.

Methods

Data Acquisition from METABRIC and TCGA

We obtained transcriptomic and clinical data of 1075 female patients who had a histopathological diagnosis of BC in TCGA cohort [9] through cBioportal (TCGA PanCancer Atlas dataset) [10, 11] and (Liu et al., 2018 dataset) [12]. We also obtained the data of 1904 patients in METABRIC cohort through cBioPortal (METABRIC Nature 2012 & Nat Commun 2016 dataset) [10] and (Rueda et al., 2019 dataset) [13] with these similar features. In METABRIC and TCGA, given that the patient data are de-identified, and that it is in a public domain, it waived Institutional Review Board approval.

Tissue samples

Formalin-Fixed Paraffin-Embedded (FFPE) tissues were obtained from 112 BC patients, who underwent breast surgery between 2005 and 2012 at Hokkaido University Hospital. Informed consent was obtained from all patients. A part of this dataset once validated the usefulness of tools for predicting the additional effects of CT given in addition to ET. The Ethics Committee of Hokkaido University Hospital (Hokkaido, Japan) approved the use of in-house data.

Extraction of RNA and preparation for analysis by expression microarray

FFPE tissues were stored until the study was performed and four sections of 10 μ m thickness of FFPE specimens were obtained for RNA extraction. RNA extraction was performed using the RNeasy FFPE Kit according to the manufacturer's protocol (Qiagen, Hilden, Germany)). The extracted RNA was synthesized into cDNA using the Ovation FFPE WTA Systems (Affymetrix, Santa Clara, CA, USA) and the Encore Biotin Module

(Affymetrix) kits according to the manufacturer's instructions. The cDNA (100ng) was then used for gene expression analysis by DNA microarray using the Human Genome U133 Plus 2.0 Array according to the manufacturer's instructions (Affymetrix).

Estimation of estrogen reactivity

GSVA score of the "Hallmark estrogen response early" and "Hallmark estrogen response late" gene sets in the MSigDB Hallmark collection [14] was used to measure the estrogen reactivity. GSVA Bioconductor package (version 3.10) was used.

Statistical analyses of RNA expression and Gene Set Enrichment Analyses (GSEA)

Analysis followed a two-step process as previously described [6, 15]. We first calculated the fold changes of genes, corresponding to a high relative to low estrogen reactivity, which provided a list of *t*-scores and corresponding *p*-values for high relative to low estrogen reactivity in relation to each of the gene's expression values. In a second step, gene set enrichment analysis was performed in Gene Set Enrichment Analyses (GSEA) Pre-ranked using these collections of gene sets from the Hallmarks gene sets using software provided by the Broad Institute (<http://software.broadinstitute.org/gsea/index.jsp>). We only considered gene sets significantly enriched that met a threshold of normalized enrichment score (NES) >1.8 or <-1.8 and false discovery rate (FDR) *q*-value < 0.01.

Tumor Immune Microenvironment Analysis

CIBERSORT deconvolution algorithm [16] was used to estimate the fraction of immune cells in the tumor microenvironment using transcriptomic data of the whole tumor via the online calculator (<https://cibersort.stanford.edu/>) as previously shown [6, 15, 17]. The immune cytolytic activity (CYT) was defined as the geometric mean of GZMA and PRF1 expression values in Transcripts Per Million [18, 19] and CYT was calculated as previously described [6, 15, 17, 20-24].

Statistical Analysis

All statistical analyses were performed using R software (<http://www.r-project.org/>) and Bioconductor (<http://bioconductor.org/>). The chi-square test or Fisher's exact test or the nonparametric Mann-Whitney U test and contingency analysis were used to assess baseline differences between binary variables. Correlations were calculated using Spearman's rank correlation coefficient. In the analysis of recurrence-free survival (RFS), overall survival (OS), and breast cancer specific survival (BCSS), the Kaplan–Meier method was used to estimate survival rates, and differences between survival curves were evaluated by the log-rank test. Two-sided *P* values <0.05 was considered as statistically significant for all tests.

Results

A total of 1904 women with BC in the METABRIC cohort, 1082 women with BC in TCGA BC cohort, and 112 women with BC in the Hokkaido University hospital cohort in which transcriptomic data associated with clinical parameters were analyzed. We investigated the clinical relevance of estrogen reactivity in these BC cohorts based on the scores of ESTROGEN_RESPONSE_EARLY and ESTROGEN_RESPONSE_LATE in GSVA.

ESTROGEN_RESPONSE_EARLY gene set contained 200 genes which react early to estrogen and ESTROGEN_RESPONSE_LATE gene set contained 200 genes which react late to estrogen. We showed that there was a strong correlation between ESTROGEN_RESPONSE_EARLY score and ESTROGEN_RESPONSE_LATE score in METABRIC (the whole, $r = 0.895$; hormone receptor (HR) + human epidermal growth factor receptor 2 (HER2)-, $r = 0.854$; HER2+, $r = 0.871$; triple negative (TN), $r = 0.751$) and in TCGA (the whole, $r = 0.889$; HR + HER2-, $r = 0.85$; HER2+, $r = 0.867$; TN, $r = 0.811$), as well as in our institutional cohort ($r = 0.948$) (**Supplementary Fig. 1**). The scores of ESTROGEN_RESPONSE_EARLY and ESTROGEN_RESPONSE_LATE were the highest in the HR + HER2- subgroup in METABRIC and TCGA (Fig. 1A). We defined the score of ESTROGEN_RESPONSE_EARLY as more than the 75th percentile for dividing the group into estrogen reactivity-high and estrogen reactivity-low groups in the HR + subgroup, which was the top 75 % (1016/1355) in the HR + HER2- in METABRIC, the top 75.2 % (440/585) in the HR + HER2- in TCGA, and the top 74.1 % (83/112) in our cohort. In METABRIC, this cutoff value was the top 56.8 % (1081/1904) of the whole cohort, the top 43.4 % (59/136) in the HER2+, and was the top 1.9 % (6/306) in the TN. In TCGA, this cutoff value was the top 57.8 % (625/1082) in the whole and was the top 51.4 % (93/181) in the HER2 + and was the top 4.7 % (8/172) in the TN. This cutoff value could dichotomize the ESTROGEN_RESPONSE_EARLY score and ESTROGEN_RESPONSE_LATE score of BC patients based on statistical significance in the whole cohort and each amongst each subtype in METABRIC, TCGA (Fig. 1B and **Supplementary Fig. 2**), and in our cohort (Fig. 1C). In immunohistochemistry (IHC) study for ER and progesterone receptor (PgR), there was no difference of percentage of ER due to estrogen reactivity, but high estrogen reactivity was statistically significantly associated with higher percentage of PgR, which is an estrogen-responsive gene (Fig. 1D). These results indicate that estrogen reactivity can be quantified utilizing ESTROGEN_RESPONSE_EARLY score in GSVA.

Association of estrogen reactivity with clinical features

We studied the relationship between clinical features of the primary tumor and the levels of estrogen reactivity in the HR + HER2- subgroup in METABRIC, TCGA (**Supplementary Table 1**), and our cohort (**Supplementary Table 2**). In METABRIC, patients with high estrogen reactivity were significantly associated with lower age ($p = 0.002$), premenopausal state ($p = 0.002$), PgR+ ($p < 0.0001$), and Luminal A subtype ($p < 0.0001$). In TCGA, patients with high estrogen reactivity were significantly associated with lower age ($p = 0.037$), premenopausal state ($p = 0.0075$), and PgR positive ($p < 0.0001$). In our cohort, patients with high estrogen reactivity were significantly associated with lower grade ($p = 0.016$). These results indicate that high estrogen reactivity was related to lower age, premenopausal state, and PgR positivity.

Gene expression profiles by the levels of estrogen reactivity

In order to clarify the mechanisms associated with the levels of estrogen reactivity, volcano plots and pre-ranked GSEA were performed in all cohorts. Figure 2 show volcano plots that represent the distribution of the fold changes and adjusted p -values of 18,484 genes in METABRIC HR + HER2- cohort, 18,428 genes in TCGA HR + HER2- cohort, and 23,372 genes in our cohort corresponding to the levels of estrogen reactivity. In the high estrogen reactivity group, 13 mRNAs in METABRIC HR + HER2- cohort, *ADCY1*, *STC2*, *TFF1*, *TUBA3D*, *GFRA1*, *SUSD3*, *MAPT*, *SLC7A2*, *CLIC6*, *SCUBE2*, *PDZK1*, *SERPINA3*, and *SLC39A6* were up-regulated (Fig. 2A). In TCGA HR + HER2- cohort, 1 mRNA, *PPFIA1*, was up-regulated and 7 mRNA, *ATCAY*, *DEFB4A*, *KRT24*, *PASD1*, *RSP02*, *SPRR2A*, and *SPRR2B*, were down-regulated (Fig. 2B). 10 mRNAs in our cohort, *C8orf44-SGK3*, *PCDHGB7*,

ABCD3, *RDH13*, *MTR*, *GREB1*, *PGR*, *FAM35A*, *ANKRD36B*, and *AGR3*, were up-regulated (Fig. 2C), all of which were differentially expressed with a fold-change greater than $\log_2(1)$ and $p < 0.05$.

Figure 3 shows pre-ranked GSEA. In high estrogen reactive group, in all cohorts, estrogen response (METABRIC: Early; NES = 3.69 FDR $q < 0.0001$ and Late; NES = 3.21 FDR $q < 0.0001$, TCGA: Early; NES = 4.13 FDR $q < 0.0001$ and Late; NES = 3.47 FDR $q < 0.0001$, and our cohort: Early; NES = 2.6 FDR $q < 0.0001$ and Late; NES = 1.85 FDR $q = 0.005$) were enriched. Additionally, in our cohort, Myc targets v1 (NES = 3.21, FDR $q < 0.0001$), Metabolism related gene sets (protein secretion; NES = 2.98, FDR $q < 0.0001$, oxidative phosphorylation; NES = 2.27, FDR $q < 0.0001$, adipogenesis; NES = 1.83, FDR $q < 0.0001$), cell-stress responsive gene sets (unfolded protein response; NES = 2.46, FDR $q < 0.0001$, UV response down; NES = 2.16, FDR $q < 0.0001$), TGF-beta signaling (NES = 2.31, FDR $q < 0.0001$), androgen response (NES = 2.21, FDR $q < 0.0001$), and MTORC1 signaling (NES = 1.96, FDR $q = 0.004$) were enriched. In low estrogen responsive group, in METABRIC and TCGA, immune-related proteins (Allograft rejection (METABRIC: NES=-3.25, FDR $q < 0.0001$, TCGA: NES=-2.48, FDR $q < 0.0001$), Interferon (IFN)- α/γ response (METABRIC: α ; NES=-2.53, FDR $q < 0.0001$, γ ; NES=-2.95, FDR $q < 0.0001$, TCGA: γ ; NES=-2.14, FDR $q < 0.0001$), Inflammatory response (METABRIC: NES=-2.32, FDR $q < 0.0001$, TCGA: NES=-2.15, FDR $q < 0.0001$), and Complement (METABRIC: NES=-2.2, FDR $q < 0.0001$, TCGA: NES=-1.88, FDR $q < 0.0001$)), IL2-STAT5 signaling (METABRIC: NES=-0.49, FDR $q < 0.0001$, TCGA: NES=-1.87, FDR $q < 0.0001$), IL6-JAK-STAT3 signaling (METABRIC: NES=-1.97, FDR $q < 0.0001$, TCGA: NES=-1.94, FDR $q < 0.0001$), and KRAS signaling up (METABRIC: NES=-2.06, FDR $q < 0.0001$, TCGA: NES=-1.96, FDR $q < 0.0001$) were enriched. Additionally, in METABRIC, cell cycle related gene sets (E2F targets; NES=-2.14, FDR $q < 0.0001$, G2M checkpoint; NES=-1.98, FDR $q < 0.0001$) and, in TCGA, epithelial mesenchymal transition (NES=-1.85, FDR $q < 0.0001$) and, in our cohort, KRAS signaling down (NES=-1.84, FDR $q < 0.0001$) were enriched. These results indicate that high estrogen reactivity was related to Myc targets, Metabolism related gene sets, TGF-beta signaling, androgen response, UV response down (stress-related pathway), and MTORC1 signaling. Low estrogen reactivity was related to immune-related proteins, IL2-STAT5 signaling, IL6-JAK-STAT3 signaling, KRAS signaling, cell cycle related gene sets, and epithelial mesenchymal transition.

High estrogen reactivity was associated with low immune cytolytic activity, low levels of immunostimulatory cells, and high levels of immunosuppressive cells.

In order to investigate the TME by estrogen reactivity, we analyzed CYT and the immune cell composition utilizing CIBERSORT in all cohorts (Fig. 3). It has been well established that CYT score represents the overall cytolytic activity of immune effector cells in a bulk tumor [18]. We found that CYT scores in BC tumors were significantly lower in high estrogen reactivity group in all cohorts. Among the immunostimulatory cells, M1 macrophages and activated NK cells were lower within high estrogen reactivity group in METABRIC cohort. CD8 + T-cells were lower in both TCGA and our cohorts. Activated CD4 + memory T-cells were lower in both METABRIC and our cohorts. Follicular helper T-cells were lower in TCGA. Among the immunosuppressive cells, M2 macrophages were higher within high estrogen reactivity group in all cohorts. Regulatory T cells (Tregs) were lower in TCGA. In terms of the other immune cells, resting MAST cells were lower within high estrogen reactivity group in all cohorts. $\Gamma\delta$ T cells were lower in both METABRIC and TCGA. Eosinophils were lower in both METABRIC and our cohorts. Monocytes were higher in TCGA and lower in our cohort. B cells were higher and plasma cells were lower in METABRIC. Activated MAST cells were lower and neutrophils were higher in our cohort. There was no correlation between tumor tissue and blood in lymphocyte, neutrophil, basophil, and

monocyte counts (**Supplementary Fig. 3**). These results indicate that patients with high estrogen reactivity had low CYT, low levels of immunostimulatory cells, and high levels of immunosuppressive cells in BC TME.

Stimulatory and inhibitory factors of the cancer-immunity cycle (CIC) were decreased in high estrogen reactive BC.

The cancer-immunity cycle (CIC) is a series of self-sustaining stepwise events required to gain efficient control of cancer growth by the immune system [25]. We reported that disability of CIC had a significant effect on TME and prognosis of BC patients [15]. Here, we explored the relationship between the levels of estrogen reactivity and stimulatory or inhibitory factors of the CIC in all cohorts (Fig. 4). In the analysis of stimulatory factors of cancer cells in the CIC, HLA-class I expression was lower in high estrogen reactivity group in all cohorts. In the analysis of inhibitory factors of cancer cells in the CIC, the expression of PD1, PD-L1, PD-L2, and IDO1 were lower in high estrogen reactivity group in all cohorts. These results indicate that stimulatory factors inhibitory factors of cancer cells were statistically significantly decreased in high estrogen reactivity group.

Patients with high estrogen reactivity were associated with better RFS in all cohorts.

In order to investigate whether estrogen reactivity can serve as a prognostic biomarker, we examined the relationship between the levels of estrogen reactivity and prognosis, which were tested by the Kaplan–Meier method and verified by the log-rank test and cox regression analysis in all cohorts (Fig. 5). In METABRIC HR + HER2- cohort, distant recurrence analysis showed patients with high estrogen reactivity were significantly associated with better prognosis (Fig. 5A, $p < 0.0001$). However, there were no significant differences between the levels of estrogen reactivity and local recurrence ($p = 0.11$). Patients with high estrogen reactivity were significantly associated with better RFS in TCGA HR + HER2- cohort ($p = 0.037$) and our cohort ($p = 0.031$). In cox regression analysis, high estrogen reactivity proved to be a significant good prognostic parameter of distant RFS (DRFS) (HR: 0.66, 95%CI: 0.53–0.82, $p = 1.63E-04$) in METABRIC HR + HER2- cohort and RFS in TCGA HR + HER2- cohort (HR: 0.56, 95%CI: 0.33–0.97, $p = 3.96E-02$) and in our cohort (HR: 0.31, 95 % CI: 0.098–0.95, $p = 0.041$) (Table 1). Despite differences in mortality rates between three cohorts, high estrogen reactivity was associated with better prognosis in the HR + subtype.

Table 1
Univariate and multivariate analysis of RFS in METABRIC and TCGA and our cohort

Univariate and multivariate analysis of RFS in METABRIC and TCGA and our cohort										
Variables		METABRIC								
		Univariate analysis					Multivariate analysis			
		Value	HR	95%CI	P-value	n	HR	95%CI	P-value	n
Estrogen reactivity	(ref = Low)	High	0.66	0.53–0.82	1.63E-04	1355	0.72	0.57–0.91	0.0069	1283
PgR	(ref = Neg)	Positive	0.71	0.58–0.88	0.0017	1355	0.85	0.68–1.08	0.18	
Age	(ref = < 50)	50≥	1.02	0.78–1.33	0.91	1355				
Menopausal state	(ref = Post)	Pre	0.98	0.75–1.29	0.91	1355				
Tumor size (cm)	(ref = 2≥)	> 2	1.9	1.54–2.36	4.36E-09	1343	1.71	1.37–2.15	2.98E-06	
Node metastasis	(ref = 0)	1+	2.03	1.65–2.50	1.71E-11	1355	1.74	1.40–2.15	5.63E-07	
Tumor Grade	(ref = 1)	2,3	2.1	1.38–3.16	4.80E-04	1294	1.83	1.19–2.79	0.00548	
Variables		TCGA								
		Univariate analysis					Multivariate analysis			
		Value	HR	95%CI	P-value	n	HR	95%CI	P-value	n
Estrogen reactivity	(ref = Low)	High	0.56	0.33–0.97	3.96E-02	583	0.58	0.33–1.01	0.053	575
PgR	(ref = Neg)	Positive	0.62	0.32–1.19	0.15	583				
Age	(ref = < 50)	50≥	1.08	0.52–1.65	0.79	583				

Abbreviations: RFS, recurrence free survival; METABRIC, Molecular Taxonomy of Breast Cancer International Consortium, TCGA, The Cancer Genome Atlas; PgR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

*It was also significance in univariate and multivariate analysis. P < .05 is considered statistically significant.

Variables		METABRIC								
		Univariate analysis					Multivariate analysis			
		Value	HR	95%CI	<i>P</i> -value	n	HR	95%CI	<i>P</i> -value	n
Menopausal state	(ref = Post)	Pre	0.92	0.51–1.65	0.78	522				
Tumor size (cm)	(ref = 2≥)	> 2	2.4	1.36–2.25	2.62E-03	583	1.96	1.09–3.54	2.50E-02	
Node metastasis	(ref = 0)	1+	2.48	1.38–4.46	2.34E-03	575	2.26	1.23–4.12	8.00E-03	
Variables		Our cohort								
		Univariate analysis					Multivariate analysis			
		Value	HR	95%CI	<i>P</i> -value	n	HR	95%CI	<i>P</i> -value	n
Estrogen reactivity	(ref = Low)	High	0.31	0.098–0.95	0.041	112	0.63	0.17–2.42	0.51	103
PgR	(ref = Neg)	Positive	0.67	0.18–2.47	0.54	112				
HER2	(ref = Neg)	Positive	1.14	0.15–8.83	0.9	109				
Ki67	(ref = < 20)	20≥	3.81	1.16–12.5	0.0279	104	3.51	0.94–13.2	0.062	
Age	(ref = < 50)	50≥	1.84	0.40–8.43	0.43	112				
Menopausal state	(ref = Post)	Pre	0.41	0.09–1.86	0.25	112				
Tumor size (cm)	(ref = 2≥)	> 2	12.6	2.73–58.4	0.0012	110	9.6	2.00–46.1	0.0047	

Abbreviations: RFS, recurrence free survival; METABRIC, Molecular Taxonomy of Breast Cancer International Consortium, TCGA, The Cancer Genome Atlas; PgR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

*It was also significance in univariate and multivariate analysis. $P < .05$ is considered statistically significant.

Variables		METABRIC								
		Univariate analysis					Multivariate analysis			
		Value	HR	95%CI	<i>P</i> -value	n	HR	95%CI	<i>P</i> -value	n
Node metastasis	(ref = 0)	1+	1.78	0.57–5.50	0.32	112				
Tumor Grade	(ref = 1)	2,3	4.83	0.62–37.8	0.13	109				
Abbreviations: RFS, recurrence free survival; METABRIC, Molecular Taxonomy of Breast Cancer International Consortium, TCGA, The Cancer Genome Atlas; PgR, progesterone receptor; HER2, human epidermal growth factor receptor 2.										
*It was also significance in univariate and multivariate analysis. P < .05 is considered statistically significant.										

The tumor microenvironment in recurrent breast cancer patients treated with chemotherapy and endocrine therapy was correlated with higher levels of cell cycle related gene sets, lower levels of immunosuppressive cells, and higher levels of CIC regulators.

ESTROGEN_RESPONSE_EARLY score is composed of genes involved in the endocrine therapeutic response, and we have shown that a low score was significantly associated with a worse response to endocrine therapy [26]. Here, we explored the relationship between the additional effect of CT given in addition to ET and signaling pathways in GSVA, CYT and immune cell composition, and CIC regulators (Fig. 6). Based on treatments and recurrence, we classified patients into following three groups: patients treated with both ET and CT but relapsed as “CT rec”, patients treated with ET alone but relapsed as “ET rec”, and patients treated with ET with or without CT and did not relapsed as “No rec”. E2F targets, G2M checkpoint, Myc targets v2, and PI3K AKT mTOR signaling GSVA scores were the highest in “CT rec” and the lowest in “No rec” in METABRIC HR + HER2- cohort. ESTROGEN_RESPONSE_EARLY score was the highest in “No rec”. Fatty acid metabolism, Protein secretion, and Xenobiotic metabolism scores were the highest in “CT rec” and the lowest in “ET rec”. In our cohort, E2F targets and G2M checkpoint scores were lower in the “No rec” group, compared with the recurrence groups. Wnt beta catenin signaling score was significantly higher in “CT rec”, compared with “ET rec”. There was no significant difference in ESTROGEN_RESPONSE_EARLY and LATE in three groups. In the analysis of the relationship between three groups in breast TME, CYT scores were the highest in “CT rec” in METABRIC, but there was no significant difference in our cohort. Regarding the immune cell composition, immunostimulatory cells, M1 macrophages were higher in “CT rec” compared with “No rec”, and follicular helper T-cells were lower in “CT rec” compared with “ET rec” in METABRIC. Immunosuppressive Tregs cells were the lowest in “CT rec”. Monocytes were higher in “CT rec” compared with “ET rec”. In our cohort, M2 macrophages were lower in “CT rec” compared with “No rec”. In the analysis of regulators in the CIC, the expression of HLA-class I was the highest in “CT rec” in METABRIC, but there was no significant difference in our cohort. The expression of PD-L1 and IDO1 were the highest in “CT rec” in METABRIC, but there was no significant difference in our cohort. These results indicate that the TME in “CT rec” was related with higher levels of E2F targets and G2M checkpoint and lower levels of immunosuppressive M2 macrophages or Tregs cells. In addition, the TME in “CT rec” had higher levels of CIC regulators.

Discussion

Estrogen signals play an important role in the molecular and genomic pathways of HR + BC phenotype by affecting the transcriptional modulation of various genes such as proliferation regulators, growth factors, cell cycle, and apoptosis modulators [3]. The physical behavior of stromal cells, extracellular matrix, immune cells, macrophages, and soluble factors can modulate tumor cells and the TME to promote cancer survival or progression and can influence therapeutic efficacy [7]. Therefore, it was of interest to investigate estrogen reactivity in the breast cancer TME. How estrogen signals effect the TME and treatment response in primary BC remains unclear. We aimed to test the hypothesis that estrogen reactivity effects immune cells and gene expression profiles in the TME, and that estrogen reactivity is related to recurrence after treatment with CT and ET using GSVA scores of ESTROGEN_RESPONSE_EARLY and ESTROGEN_RESPONSE_LATE on our institutional cohort and collected data from METABRIC and TCGA BC cohorts. This study generated four interesting results with clinical implications.

First, high estrogen reactivity was related to Myc targets, Metabolism related gene sets, cell-stress responsive gene sets, TGF-beta signaling, androgen response, and MTORC1 signaling. Low estrogen reactivity was related to immune-related proteins, IL2-STAT5 signaling, IL6-JAK-STAT3 signaling, KRAS signaling, cell cycle related gene sets, and EMT (Fig. 2). Some pathways that have been found to be related to the degree of estrogen sensitivity have been reported as follows: Oxidative phosphorylation is regulated via ER β in TNBC [27] and adipogenesis pathway induces higher estrogen reactive TME thus enabling mammary tumorigenesis [28]. Unfolded protein response signaling promotes a malignant phenotype in BC and can confer tumors with resistance to widely used therapies [29]. There is a direct link between mTORC1 and ER α , which further implicates mTORC1 signaling in the pathogenesis of ER-positive BC [30]. In the cell cycle, although the molecular mechanism underlying estrogenic regulation of G1 phase is unclear, current evidence suggests that estrogens may regulate some important molecules required for transition to S phase [31]. Decreased estrogen signaling has been reported to result in potent EMT characterized by significant changes in the expression profile of certain matrix macromolecules [32].

Second, BC with high levels of estrogen reactivity has low immune cytolytic activity, low levels of immunostimulatory cells, and high levels of immunosuppressive cells (Fig. 3). It also has low levels of stimulatory and inhibitory factors of the CIC (Fig. 4). In GSEA, we confirmed that the degree of estrogen reactivity was related to immune-related proteins (Fig. 2). It has been reported that both genomic and non-genomic estrogen signals have been implicated in several effects of immunity and autoimmunity [33, 34]. These studies further showed that estrogen signals effect the size, maturation, and development of T cells. In CD4 + T cells, estrogen signals are associated with the inhibition of the production of Th1 pro-inflammatory cytokines and the promotion of Th2 anti-inflammatory cytokines. E2 through the ER α pathway also impacts the activation and survival of B cells. Aside from lymphocytes, estrogen exposure delays apoptosis in human neutrophils, causes a reduction in NK cell cytotoxic activity, and modulates different macrophage actions and their metabolism. We showed that low expressions of estrogen responsive genes were correlated with the presence of lymphocytes with cytolytic activity [15]. In summary, lymphocytes, fibroblasts, neutrophils, eosinophils, macrophages, and myeloid-derived suppressor cells present in the TME are disturbed by estrogen signaling, which effects the growth and migration of cancer cells. Further, it has been revealed that the immune response in tumor tissue reflects a series of carefully regulated events that can be optimally addressed as a

group rather than individual cells [25]. CIC is defined as a series of self-sustaining stepwise events required to gain efficient control of cancer growth by the immune system [25] and the association between CIC and estrogen signaling remains unknown. We showed that high estrogen sensitivity was correlated with CIC dysfunction [15]. These results demonstrate that high estrogen reactivity not only reduces the distribution of anti-tumor immune cells in the TME, but also inactivates the anti-tumor immune system.

Third, patients with high estrogen reactivity were associated with a better prognosis. We showed that, despite differences in mortality rates between three cohorts, high estrogen reactivity was associated with better prognosis in the HR + subtype (Fig. 5 and Table 1). This result is reasonable as shown in prior reports. Perou and colleagues have been reported that the estrogen-induced gene set identified in MCF-7 cells was used to hierarchically cluster a 65 tumor training set into two groups, showing a significant differences in survival [35]. The group with a better prognosis showed high expression of estrogen- and GATA3-regulated genes. Recently, we reported that the ESTROGEN_RESPONSE_EARLY score was associated with survival in primary and metastatic BC [26]. On the other hand, there was no significant difference between the levels of estrogen reactivity and local recurrence (Fig. 5 and S4). These results demonstrate that high estrogen reactivity is a prognostic factor, and can be a means of predicting distant metastases.

Finally, the TME in CT rec was related with higher levels of E2F targets and G2M checkpoint and lower levels of immunosuppressive M2 macrophages and Tregs cells. In addition, the TME in CT rec had higher levels of CIC regulators. According to a widely accepted idea, not only cancer cell-specific properties, but also signals derived from cells in TME play critical roles in the response to cancer therapy [7]. For example, Rosenfeldt and his colleagues showed that the E2F1–ABCG2 axis suppresses CT-induced cell death that can be restored by the inhibition of ABCG2 [36]. Further, Velaei and colleagues reviewed that the impact of conventional anticancer CT on the relationship between the tumor and the immune system as follows [7]: High levels of the tumor-infiltrating lymphocytes (TILs) in the ER-HER2 + subtype are related with good response to CT. Above all, high levels of CD8 + cytotoxic T lymphocytes synergistically increase the effect of anthracycline or anthracycline/taxane based neoadjuvant CT and low levels of Tregs enhance CT response. In addition, targeting tumor-associated macrophage in combination with CT can improve the effect of CT since macrophages induced by cytotoxic CT can protect tumor cells from death due to a cathepsin-dependent function. Interestingly, the levels of estrogen reactivity were not a predictor of therapeutic benefit of CT given in addition to ET. Our cohort once validated the usefulness of tools for predicting the additional effects of CT on ET. Therefore, our cohort seems to be more suitable for this analysis and these results seem to be credible. These findings demonstrate that cell cycle signaling and the immunosuppressive TME, not estrogen reactivity, play a pivotal role in cancer response to additional CT given in addition to ET.

Although the study demonstrates promising results, it has limitations. Although we utilized multiple large patient cohorts, this is a retrospective study utilizing our own institutional data and publicly available datasets. Second, each cohort we survey has different patient backgrounds and clinical characteristics. In METABRIC and TCGA, some of the clinical factors deemed necessary were missing. TCGA cohort is known to lack co-morbidities and therapeutic intervention data. Our institutional cohort has sufficient clinical data, but a small number of patients. Therefore, it is necessary to verify the findings obtained with other cohorts with sufficient clinical data. Third, we could not investigate the association of the estrogen reactivity with the effects of drugs

used in recurrent BC, especially CDK4/6 inhibitors. Finally, this study does not include in vitro or in vivo experiments. Therefore, the mechanism for further understanding the results has not been clarified.

Conclusions

We demonstrated the relationship between estrogen reactivity and immune cells and gene expression profiles in the TME. We also demonstrated the therapeutic benefit of CT given in addition to ET utilizing our institutional cohort as well as METABRIC and TCGA BC cohorts. We revealed that estrogen reactivity perturbs multiple pathways in the TME. High estrogen reactivity acts suppressively against the immune microenvironment and acts as a prognostic factor. In addition, higher levels of E2F targets and G2M checkpoint gene sets, but lower levels of immunosuppressive cells may be a biomarker that can be used to decide whether to add CT to ET in the adjuvant setting. Based on these reported results, we anticipate that further research can be conducted to establish a greater understanding of the role of estrogen reactivity to TME of BC.

Abbreviations

BC, breast cancer; ER+, estrogen receptor positive; E2, estradiol; TME, tumor microenvironment; GSVA; Gene Set Variant Analysis; CT, chemotherapy; ET, endocrine therapy; METABRIC, Molecular Taxonomy of Breast Cancer International Consortium; TCGA, the cancer genome atlas; FFPE, Formalin-Fixed Paraffin-Embedded; GSEA, Gene Set Enrichment Analyses; NES, normalized enrichment score; FDR, false discovery rate; CYT, immune cytolytic activity; RFS, recurrence free survival; OS, overall survival; BCSS, breast cancer specific survival; HR, hormone receptor; HER2, human epidermal growth factor receptor 2; TN, triple negative; IHC, immunohistochemistry; PgR, progesterone receptor; IFN, interferon; Tregs, regulatory T cells; CIC, cancer-immunity cycle; DRFS, distant RFS; TILs, the tumor-infiltrating lymphocytes.

Declarations

Ethics approval and consent to participate

This study was approved by the Medical Ethics Committee, Hokkaido University Hospital and written consent was obtained from all participants.

Consent for publication

All authors have agreed to publish this manuscript.

Availability of supporting data

All data generated or analyzed during this study are included either in this article or in the supplementary files.

Competing interests

All of the authors declare that they have no actual, potential, or perceived conflict of interest regarding the manuscript submitted for review.

Funding

This work was supported by NIH grant R01CA160688 to K.T, and NCI grant P30CA016056 involving the use of Roswell Park Cancer Institute's Bioinformatics and Biostatistics Shared Resources.

Authors' contributions:

T.T. conceptualized and performed analysis and prepared the article.

T.T., T.O., Y.T., M.O., and W.T. performed analysis.

Y.H. and K.CH. provided the data.

T.T. provided supervision and prepared the article.

L.Y. supervised analysis

A.P. and K.T. prepared the article.

Acknowledgments

Not applicable

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Figures

Figure 1

The association of estrogen response scores in GSVA with breast cancer subtype, estrogen reactivity, and immunohistochemistry for ER and PgR. Boxplots of the comparison of estrogen response early and late score

by subtype; HR+/ HER2-, HER2+, and TN (A), and by estrogen reactivity in METABRIC and TCGA cohorts (B) and our cohort (C) were shown. Boxplots of the comparison of ER and PgR percentage by estrogen reactivity in our cohort (D) were shown. ESTROGEN_RESPONSE_EARLY of more than 75th percentile for dividing the group into estrogen reactivity-high and estrogen reactivity-low groups in the HR+ subgroup. **** means $P < 0.0001$ and * means $P < 0.05$ Abbreviations: GSVA, Gene Set Variant Analysis; ER, estrogen receptor; PgR, progesterone receptor; METABRIC, Molecular Taxonomy of Breast Cancer International Consortium; TCGA, The Cancer Genome Atlas; HR, hormone receptor; HER2, human epidermal growth receptor 2; TN, triple negative.

Figure 2

Gene expression profiles in the levels of estrogen reactivity. Volcano plots illustrating the differentially expressed mRNAs of BC and pre-ranked GSEA of BC patients comparing high vs low estrogen reactivity in METABRIC cohort (A) and TCGA BC cohort (B) and our institutional cohort (C). left panels: In volcano plots, X-axes: log2 FC; Y-axes: $-\log_{10}$ P-value from limma analysis. mRNAs with P-value < 0.05 and FC > 1.5 are marked in red, with P-value < 0.05 and FC < 1/1.5 in green, all others in black. Right panels: In pre-ranked GSEA, orange bar shows NES and the shading of it show $-\log_{10}$ FDR q-value. We only considered gene sets significantly enriched that met a threshold of NES >1.8 or <-1.8 and FDR q-value < 0.01. ESTROGEN_RESPONSE_EARLY of more than 75th percentile for dividing the group into estrogen reactivity-high and estrogen reactivity-low groups in the HR+ subgroup. Abbreviations: BC, breast cancer; GSEA, Gene Set Enrichment Analyses; METABRIC, Molecular Taxonomy of Breast Cancer International Consortium; TCGA, The Cancer Genome Atlas; FC, fold change; NES, normalized enrichment score; FDR, false discovery rate; HR, hormone receptor.

Figure 3

Verification of the relationship between estrogen reactivity and CYT and immune cell fractions. Box plots of the relationship between estrogen reactivity and CYT; left panel and immune cell fractions; right panel in METABRIC, A, and TCGA, B, and our cohort, C were shown. ESTROGEN_RESPONSE_EARLY of more than 75th percentile for dividing the group into estrogen reactivity-high and estrogen reactivity-low groups in the HR+ subgroup. **** means $P < 0.0001$, *** means $P < 0.001$, ** means $P < 0.01$ and * means $P < 0.05$. Abbreviations: CYT, immune cytolytic activity; METABRIC, Molecular Taxonomy of Breast Cancer International Consortium;

TCGA, The Cancer Genome Atlas; HR, hormone receptor; M1, M1 macrophage; NK, activated NK cells; M2, M2 macrophage; Tfh, follicular helper cells; Tregs, CD4+ regulatory T cells.

Figure 4

Stimulatory and inhibitory factors of the CIC in the levels of estrogen reactivity. Box plots of the relationship between estrogen reactivity and stimulatory (HLA-A mRNA) or inhibitory factors (PD-1, PD-L1, PD-L2, and IDO1) of the CIC in METABRIC, A, and TCGA, B, and our cohort, C were shown. ESTROGEN_RESPONSE_EARLY of more than 75th percentile for dividing the group into estrogen reactivity-high and estrogen reactivity-low groups in the HR+ subgroup. **** means $P < 0.0001$, ** means $P < 0.01$, and * means $P < 0.05$. Abbreviations: CIC, cancer

immunity cycle; METABRIC, Molecular Taxonomy of Breast Cancer International Consortium; TCGA, The Cancer Genome Atlas; NS, not significant.

Figure 5

Analysis of the relationship between estrogen reactivity and survival in METABRIC and TCGA and our cohort. Kaplan-Meier plots of the association of estrogen reactivity with distant RFS in METABRIC (A) and RFS in TCGA (B) and in our cohort (C). ESTROGEN_RESPONSE_EARLY of more than 75th percentile for dividing the group into estrogen reactivity-high and estrogen reactivity-low groups in the HR+ subgroup. Abbreviations: RES,

recurrence-free survival; METABRIC, Molecular Taxonomy of Breast Cancer International Consortium; TCGA, The Cancer Genome Atlas; RFS, recurrent-free survival.

Figure 6

Analysis of the tumor microenvironment with no additional effect of chemotherapy & endocrine therapy in METABRIC and our cohort. Box plots of the relationship between recurrence by treatments and signaling pathways in GSVA (A) and CYT (B) and immune cell composition (C) and CIC regulators (D) in METABRIC; left, and our cohort; right. Based on treatments and recurrence, we classified patients into following three categories: a group of patients who were treated with ET and CT but relapsed as CT rec, a group of patients who were

treated with ET alone but relapsed as ET rec, and a group of patients who were treated with ET with or without CT and not relapsed as No rec. Abbreviations: METABRIC, Molecular Taxonomy of Breast Cancer International Consortium; GSVA, Gene Set Variant Analysis; CYT, immune cytolytic activity; CIC, cancer immunity cycle; ET, endocrine therapy; CT, chemotherapy; E2F, E2F_TARGETS; G2M, G2M_CHECKPOINT; Myc2, MYC_TARGETS_v2; PI3K, PI3K_AKT_MTOR_SIGNALING; FA, FATTY_ACID_METABOLISM; PS, PROTEIN_SECRETION; XM, XENOBIOTIC_METABOLISM; ERE, ESTROGEN_RESPONSE_EARLY; ERL, ESTROGEN_RESPONSE_LATE; WNT β , WNT_BETA_CATENIN_SIGNALING; M1, M1 macrophage; Tfh, follicular helper cells; M2, M2 macrophage; Tregs, CD4+ regulatory T cells.

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