

Original Article

Profile of differentially expressed intratumoral cytokines to predict the immune-polarizing side effects of tamoxifen in breast cancer treatment

Bailiang Li¹, Yang Li², Xiao-Yu Wang¹, Zi-Qiao Yan¹, Huidi Liu¹, Gui-Rong Liu¹, Shu-Lin Liu^{1,3}

¹Genomics Research Center (One of The State-Province Key Laboratories of Biomedicine-Pharmaceutics of China), Harbin Medical University, China; ²College of Bioinformatics Science and Technology, Harbin Medical University, Harbin, China; ³Department of Microbiology, Immunology and Infectious Diseases, University of Calgary, Calgary, Canada

Received November 15, 2014; Accepted January 20, 2015; Epub January 15, 2015; Published February 1, 2015

Abstract: Factors within the tissue of breast cancer (BC) may shift the polarization of CD4+ T cells towards Th2 direction. This tendency can promote tumor development and be enhanced by the use of tamoxifen during the treatment. Thus, the patients with low levels of tumor-induced Th2 polarization prior to tamoxifen treatment may better endure the immune-polarizing side effects (IPSE) of tamoxifen and have better prognoses. Estimation of Th2 polarization status should help predict the IPSE among tamoxifen-treated patients and guide the use of tamoxifen among all BC patients before the tamoxifen therapy. Here, we report profiling of differentially expressed (DE) intratumoral cytokines as a signature to evaluate the IPSE of tamoxifen. The DE genes of intratumoral CD4+ T cells (CD4 DEGs) were identified by gene expression profiles of purified CD4+ T cells from BC patients and validated by profiling of cultured intratumoral CD4+ T cells. Functional enrichment analyses showed a directed Th2 polarization of intratumoral CD4+ T cells. To find the factors inducing the Th2 polarization of CD4+ T cells, we identified 995 common DE genes of bulk BC tissues (BC DEGs) by integrating five independent datasets. Five DE cytokines observed in bulk BC tissues with dysregulated receptors in the intratumoral CD4+ T cells were selected as the predictor of the IPSE of tamoxifen. The patients predicted to suffer low IPSE (low Th2 polarization) had a significantly lower distant relapse risk than the patients predicted to suffer high IPSE in independent datasets ($n = 608$; $HR = 4.326$, $P = 0.000897$; $HR = 2.014$, $P = 0.0173$; $HR = 2.72$, $P = 0.04077$). Patients predicted to suffer low IPSE would benefit from tamoxifen treatment ($HR = 2.908$, $P = 0.03905$). The DE intratumoral cytokines identified in this study may help predict the IPSE of tamoxifen and justify the use of tamoxifen in BC treatment.

Keywords: Breast cancer, tamoxifen, immune-polarizing side effects, the CD4+ T cell, gene expression profiles, cytokine

Introduction

It is increasingly recognized that human cancers *in vivo* are not homogeneous collections of neoplastic cells but rather consist of complex multicellular systems [1]. The interplay between tumor cells and stroma has been demonstrated to be a vital factor in cancer biologies [2] and responses to different therapeutics [1]. Infiltrating immune cells, which exert diverse biological functions [3], are frequently observed in all types of breast cancer (BC) [4, 5]. For example, CD4+ T cells are an integral part or 'regulator' in the activation and regulation processes of the host responses to many patho-

gens and cancers. Of particular interest, subtype polarization of CD4+ T cells (Th1 vs. Th2 polarization) has been reported to play a decisive role in tumor immunity [6, 7]. Without the Th1 helper function of CD4+ T cells, it would be impossible for CD8+ or cytotoxic T cells (CTLs) to be competent to kill tumor cells [8]. Numerous lines of evidence have accumulated, showing that the Th2 polarization of CD4+ T cells promotes tumor development and metastasis [9-12]. Additionally, a considerable body of literature illustrates that the CD4+ T cells within the human BC microenvironment may be shifted towards a Th2 phenotype to foster cancer development [10-12]. For instance, in compari-

A cytokine predictor for side effects of tamoxifen

Table 1. Basic information of datasets to develop the IPSE predictor

	Accession number	No. of profiles (Normal)	No. of profiles (BC)	Platform
Profiles of purified CD4+ T cells	GSE36765		10 (tumor infiltrating); 10 (blood from BC patients)	Affymetrix Human Genome U133 Plus 2.0
	GSE36767	1 (24 h); 1 (0 h)	2 (24 h); 2 (0 h)	
	GSE10780	143	42	
	GSE42568	17	104	
Profiles of bulk BC/normal tissues	GSE10810	27	31	
	GSE29431	12	54	
	GSE5764	20	10	

IPSE, immune-polarizing side effects.

A cytokine predictor for side effects of tamoxifen

Table 2. Clinical characteristics of patients in the validation cohort

	GSE6532		GSE17705	GSE12093	GSE6532
	Plus2	U133A	MDA	-	U133A untreated
Sub-datasets	Plus2	U133A	MDA	-	U133A untreated
Adjuvant therapy	Tamoxifen	Tamoxifen	Tamoxifen	Tamoxifen	None
No. of patients	87	190	195	136	86
Age					
< 65	53 (61)	85 (45)	-	-	79 (92)
≥ 65	34 (39)	96 (50)	-	-	7 (8)
Unknown	0	9 (5)	195 (100)	136 (100)	0
Grade					
I	17 (19)	33 (18)	43 (22)	8 (6)	29 (34)
II	37 (44)	94 (49)	108 (56)	43 (32)	31 (36)
III	16 (18)	31 (16)	42 (21)	30 (22)	12 (14)
Unknown	17 (19)	32 (17)	2 (1)	55 (40)	14 (16)
Size					
> 2	44 (51)	114 (60)	111 (57)	72 (53)	31 (36)
≤ 2	43 (49)	67 (35)	84 (43)	63 (46)	55 (64)
Unknown	0	9 (5)	0	1 (1)	0
Nodal status					
Positive	58 (67)	48 (25)	80 (41)	0	86 (100)
Negative	29 (33)	87 (46)	110 (56)	136 (100)	0
Unknown	0	18 (9)	5 (3)	0	0

son with non-cancerous adjacent normal tissues, the immunity within tumors was shifted towards Th2 and characterized by increased presence of B cells and CD4+ T cells [10]. CD4+ T cells isolated from human BC produce high levels of type II helper (Th2) cytokines including IL-4 and IL-13 [11, 13].

Despite its anti-tumor effects, tamoxifen, an adjuvant endocrine therapy for early-stage and estrogen-sensitive BC patients, has been reported to reduce the anti-tumor responses of CD4+ T cells in BC patients [14]. Specifically, it facilitates the shift of CD4+ T cells from Th1 to Th2 immunity, devastating the anti-tumor effect of the immune system [15, 16]. The immune shift represents a significant step in fostering a pro-tumor environment and may limit the anti-tumor effects of tamoxifen [17]. Interestingly, the immunomodulatory effects of tamoxifen appear to be independent of the estrogen-receptor (ER) [18, 19], which is the pharmacological target of tamoxifen.

Our postulation is that low levels of tumor-induced Th2 polarization within the tumor microenvironment may help circumvent the CD4+ T cell-polarizing effects of tamoxifen to

certain extent and thus correspond to low levels of the immune-polarizing side effects (IPSE) of tamoxifen. As a result, tamoxifen-treated patients with lower IPSE (Th2 polarization) could have better prognoses. In this study, by integrating gene expression profiles of purified CD4+ T cells and bulk BC tissues, we identified five differentially expressed (DE) cytokines and used them to assess the Th2 polarization status of intratumoral CD4+ T cells and the status of IPSE of BC patients. The ability of those five cytokines to predict the IPSE of tamoxifen is validated by survival analyses on the validation cohort. Our study might lead to novel insights into the mechanisms of the

immune shift of the intratumoral CD4+ T cells and improvement of tamoxifen therapy.

Materials and methods

Data collection and preprocessing

All the raw gene expression data were downloaded from the Gene Expression Omnibus data repository (GEO, <http://www.ncbi.nlm.nih.gov/geo/>). **Table 1** shows basic information of the gene expression profiles of purified CD4+ T cells, the BC tissues and the normal tissues, which were used to select the predictors. In GSE36767, CD4+ T cells from the tumors of two BC patients and the blood of one healthy donor were profiled immediately following isolation or were incubated for 24 h without stimulation in X-VIVO 20 (Lonza) before being profiled. Details of purification, culturing, and profiling of the CD4+ T cells can be found in [20].

The validation cohort comprises gene expression profiles of bulk BC tissues from 694 patients with the information of distant relapse-free survival (DRFS) (**Table 2**). Datasets were further divided into sub-datasets based on the profiling laboratory, therapeutic strategy and the profiling platform.

A cytokine predictor for side effects of tamoxifen

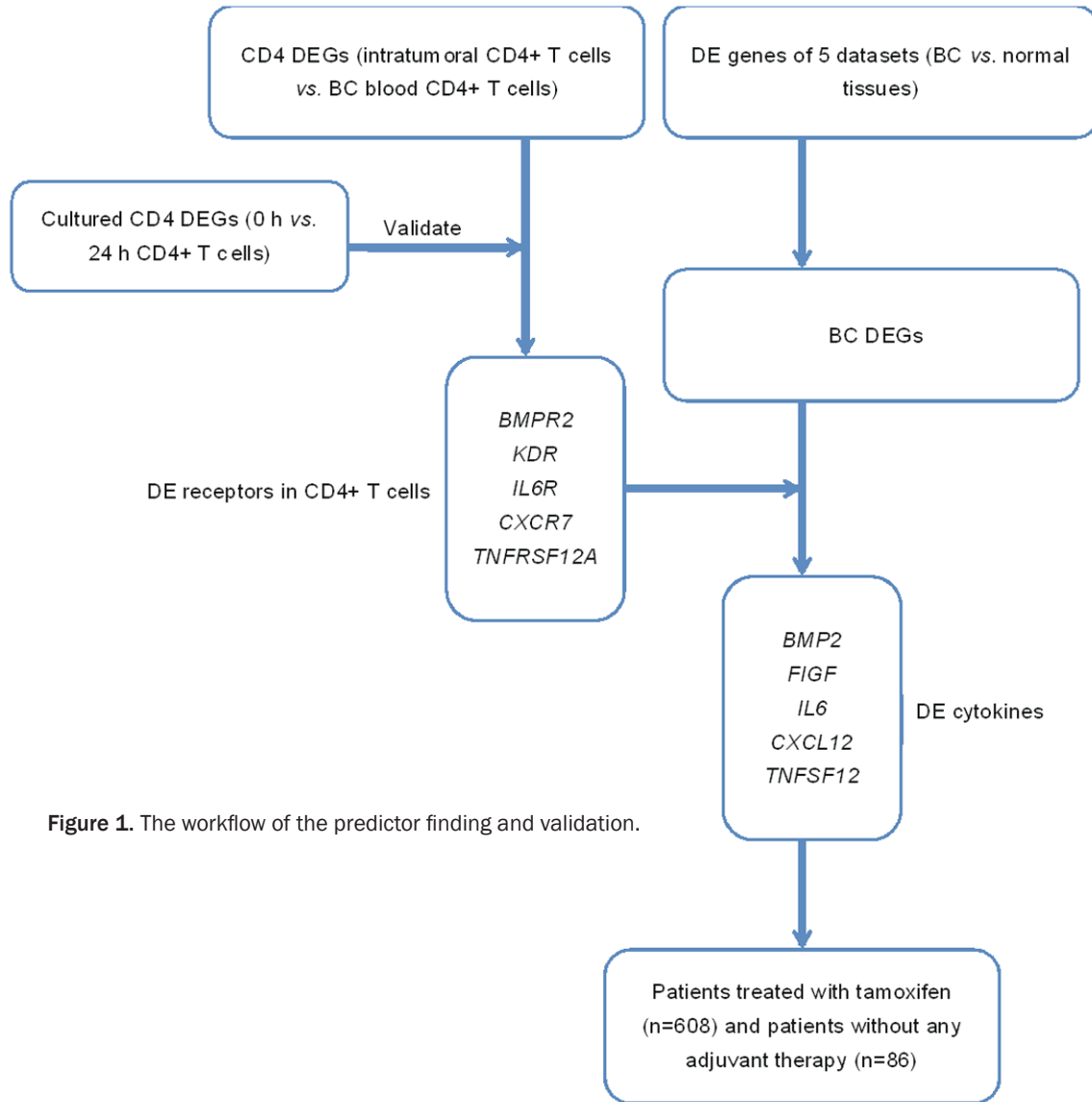


Figure 1. The workflow of the predictor finding and validation.

All datasets were normalized by the standard quantile normalization method (RMA) [21]. Probe sets that did not match any known Entrez Gene ID or matched multiple Entrez Gene IDs were abandoned. For each sample, the expression values of the probe sets matching the same gene ID were averaged as the expression value of that gene. The DE genes of GSE36767 were calculated by fold change. So except GSE36767, all expression profiles were log₂ transformed. After processing, there were 12752 and 20283 genes for the Affymetrix Human Genome U133A and U133 Plus 2.0 platforms, respectively. All genes in the Affymetrix Human Genome U133A platform were profiled in the Affymetrix Human Genome U133 Plus 2.0 platform.

Information on cytokines and their corresponding receptors was documented in the Kyoto Encyclopedia of Genes and Genomes (KEGG) website.

Identification of DE genes

The CD4 DEGs were the list of DE genes between the intratumoral CD4+ T cells and the CD4+ T cells from the blood of BC patients. The CD4 DEGs and five (GSE10780, GSE42568, GSE10810, GSE29431, GSE5764) sets of DE genes (BC vs. normal tissues) were identified by Significance Analysis of Microarrays (SAM) method (samr_2.0 R package, impute 1.32.0), with the false discovery rate (FDR) being controlled at a given level by permutations [22, 23].

A cytokine predictor for side effects of tamoxifen

Fold change values (FC) of all the profiled genes between the immediately isolated CD4⁺ T cells (0 h) and the CD4⁺ T cells cultured for 24 h without the stimuli of the microenvironment (24 h) were calculated. Genes with FC > threshold (1.5, 2, 2.5, 3) were defined as DE genes. The cultured CD4 DEGs were the list of DE genes, which were caused by culturing without the tumor microenvironment. The cultured CD4 DEGs included those genes dysregulated with the same dysregulation direction in both replicates of the cultured intratumoral CD4⁺ T cells but did not dysregulate in the CD4⁺ T cells from the healthy donor (FC ≤ 1.5).

Enrichment analysis

The functional enrichment analysis of DE genes was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 [24, 25]. The biological processes (BPs) within Gene Ontology (GO) were checked for overrepresented function entities ($P < 0.01$).

Validation of the reproducibility between DE gene sets

n_1 represents the number of DE genes with the same dysregulation direction between two DE gene sets. n_2 represents the number of all overlapped DE genes between those two DE gene sets. We used the percentage ($n_1/n_2 \times 100\%$) to test the reproducibility between two DE gene sets [26, 27]. The significance was defined by binomial distribution with prior probability of 50% for each overlapped gene to be of the same direction by chance [28].

Gene selection as the predictor of IPSE of tamoxifen

Figure 1 illustrates the workflow of the predictor selection and validation. First, the reproducibility between the CD4 DEGs and the cultured CD4 DEGs was tested at different significance levels. Based on the high reproducibility between those DE gene sets, we used the CD4 DEGs ($FDR < 0.001$) as the molecular clue about the malfunctions of intratumoral CD4⁺ T cells. The BC microenvironment could subvert the CD4⁺ T cells towards a Th2 polarization [10-12]. Therefore, we aimed to find the common and overall gene expression changes within the bulk BC tissues (BC DEGs). We performed pairwise comparisons of reproducibility among the five DE gene sets (BC tissues vs. normal tissues). Since the DE gene sets of the five datasets

were quite consistent, we defined the BC DEGs as the genes dysregulated ($FDR < 0.001$) in at least four datasets with the same dysregulation direction and, at the same time, these selected genes did not have the opposite dysregulation direction in the remaining datasets. Cytokines within the tumor microenvironment play a decisive role in the differentiation of polarized CD4⁺ T cells [7]. We used five DE cytokines observed in the BC DEGs, the receptors of which were found in the CD4 DEGs ($FDR < 0.001$), as the predictor of the Th2 polarization status of the CD4⁺ T cells and the IPSE of tamoxifen. The predictive ability was validated by assessing the correlation of the predicted IPSE with the DRFS of BC patients.

Survival analysis

Patients in the validation cohort were stratified into the low or high IPSE (Th2 polarization) groups by 2-means clustering method. Log-rank tests were used to assess the differences between the Kaplan-Meier estimates of the DRFS in the predicted groups. Multivariate survival analysis with calculation of hazard ratios (HR) and 95% confidence interval (95% CI) was performed using the Cox proportional hazards model. The examined factors included in the multivariate analysis were age (≥ 65 vs. < 65), grade (3 vs. 1 or 2), size (> 2 cm vs. ≤ 2 cm), nodal status (positive vs. negative) and the predicted IPSE status (low vs. high). The statistical significance was defined as P value < 0.05 and the marginal significance was defined as P value < 0.1 .

All statistical computations were performed in R version 2.15.3.

Results

Increased Th2 polarization of the CD4⁺ T cells in the BC environment

To investigate the gene expression pattern of the CD4⁺ T cells in BC patients, we assessed the genes dysregulated between the intratumoral CD4⁺ T cells and the CD4⁺ T cells from BC blood (CD4 DEGs). We found vast gene expression changes (8821 at $FDR < 0.1$, 6969 at $FDR < 0.05$, 4187 at $FDR < 0.01$ and 2129 at $FDR < 0.001$). Culturing in absence of the tumor microenvironment also led to fluctuation at the gene expression level. In dataset, GSE36767, the CD4⁺ T cells from the tumors of two BC patients and the blood of one healthy

A cytokine predictor for side effects of tamoxifen

Table 3. Reproducibility between CD4 DEGs and cultured CD4 DEGs

		CD4 DEGs			
		<i>FDR</i> < 0.1	<i>FDR</i> < 0.05	<i>FDR</i> < 0.01	<i>FDR</i> < 0.001
Cultured CD4 DEGs	FC > 1.5	72.58% (225/310)	74.91% (206/275)	76.42% (162/212)	80.15% (109/136)
	FC > 2	74.02% (94/127)	74.79% (89/119)	79.80% (79/99)	83.82% (57/68)
	FC > 2.5	81.33% (61/75)	83.10% (59/71)	89.66% (52/58)	89.74% (35/39)
	FC > 3	81.63% (40/49)	82.61% (38/46)	92.11% (35/38)	95.45% (21/22)

FC, fold change.

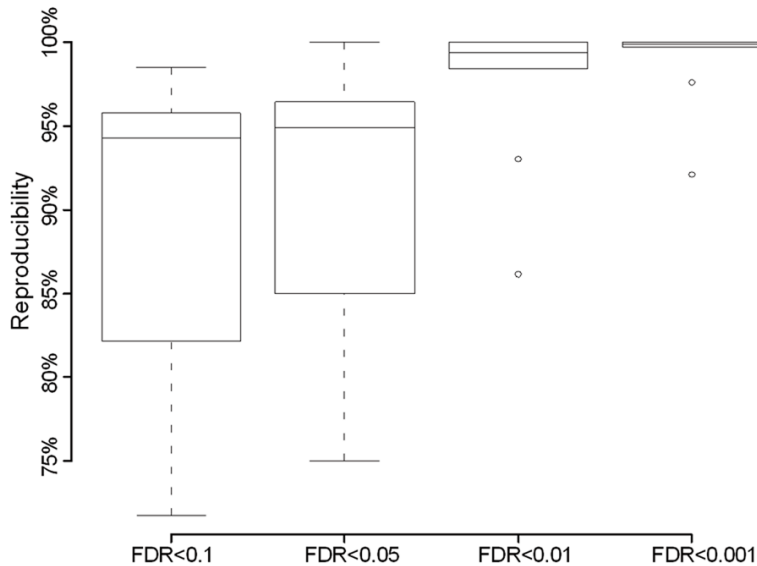


Figure 2. Boxplot of reproducibility of five DE gene sets (BC vs. normal tissues) at different significant levels.

donor were immediately profiled following isolation or were incubated without tumor stimuli for 24 h before being profiled. We identified DE genes of the intratumoral CD4⁺ T cells induced by 24-hour culturing (the cultured CD4 DEGs) by fold change and compared them with the CD4 DEGs to validate the CD4 DEGs. We observed a high reproducibility (72.58%) even at the lowest significant level ($P = 4.4 \times 10^{-16}$, **Table 3**). With the significance threshold increasing, the reproducibility between those two DE gene sets improved to 95.45%. The high reproducibility indicated that the CD4 DEGs could represent the gene expression changes of the CD4⁺ T cells, which were induced by the BC microenvironment.

To evaluate the functional status of the intratumoral CD4⁺ T cells, we conducted functional enrichment analyses on the CD4 DEGs ($FDR < 0.001$, **Online Table 1**). Consistent with the literature [10-13], our results showed that functions of the CD4⁺ T cells related to B cell activa-

tion and inflammation might be enhanced with more up-regulated genes, such as the regulation of B cell activation ($P = 0.0061$; up vs. down regulated genes: 8 vs. 6), and inflammatory responses ($P = 0.00015$; 48 vs. 14). On the other hand, the activation-related functions to other cells showed suppressed functions, such as T cell activation ($P = 0.00198$; 7 vs. 21). These results indicated that the Th2 polarization of intratumoral CD4⁺ T cells and an inflammatory environment [3, 29] might be induced within the tumor microenvironment. An immune tolerance to neoplasm might be fostered via the CD4⁺ T cells [30].

Taken together, the CD4 DEGs (**Online Table 2**) may provide molecular clues about the Th2 polarization of CD4⁺ T cells within the BC microenvironment.

Identification of BC DEGs

An enhanced Th2 polarization of the intratumoral CD4⁺ T cells is a general strategy for tumor cells to subvert the anti-tumor immunity. Thus, in order to find the cause of Th2 polarization of the intratumoral CD4⁺ T cells, we needed to identify the common gene expression changes occurring within the bulk BC tissues (BC DEGs). Cancer cells embed in and are surrounded by complex microenvironment milieu that contains various nonmalignant cells, such as inflammatory cells. As a result, traditional sampling methods can hardly avoid heterogeneity of cells or tissues taken [31]. However, this kind of heterogeneity of gene expression profiles conversely makes it feasible to investigate the overall gene expression changes with-

A cytokine predictor for side effects of tamoxifen

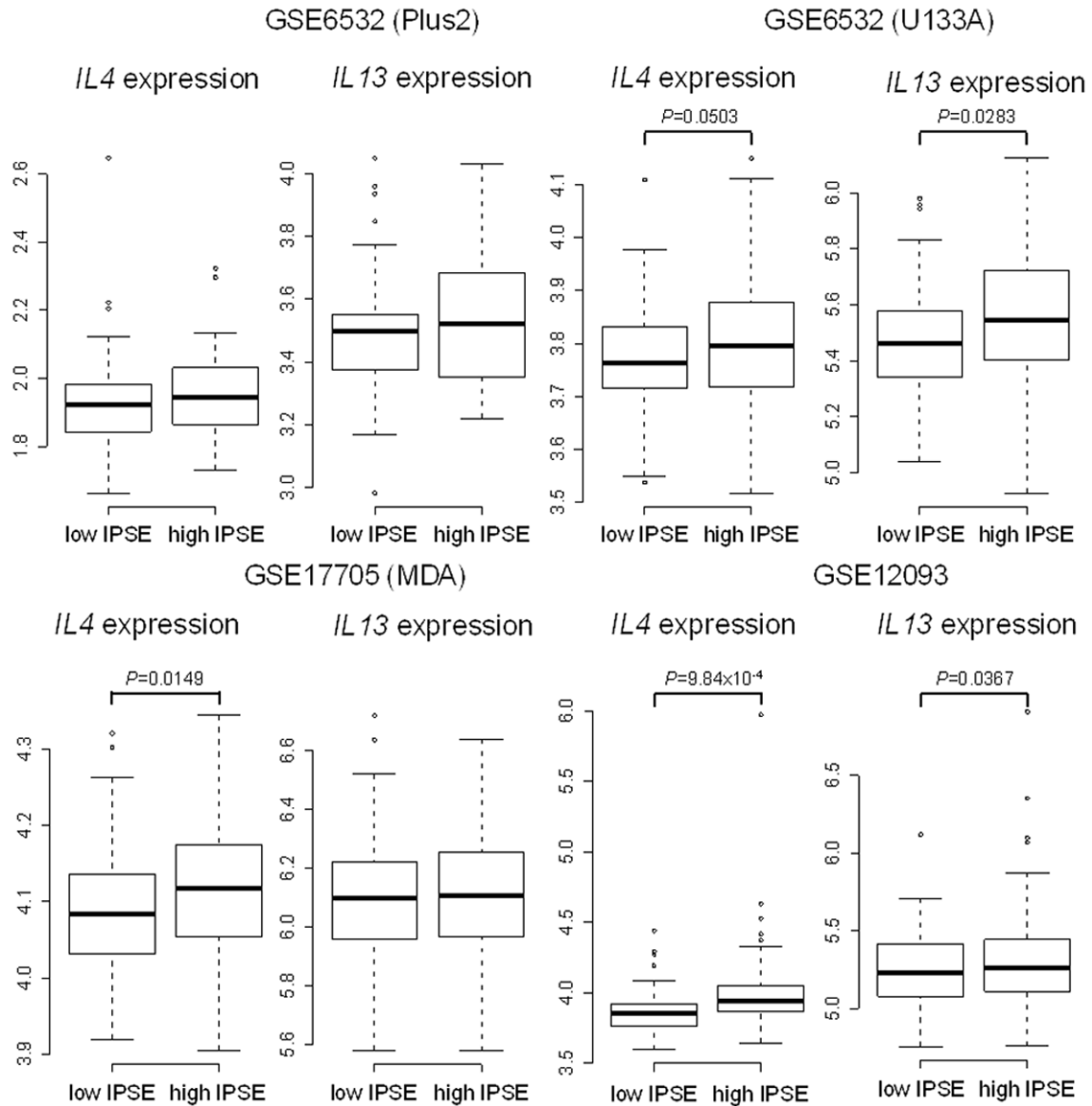


Figure 3. *IL4* and *IL13* expression levels in the validation cohort. *P* values are calculated by student t test.

in the tumor milieu [32], hence facilitating our study.

The DE genes between BC and normal tissues were identified by SAM in five independent datasets encompassing 219 normal tissues and 241 tumor tissues in total. Pairwise comparisons of the reproducibility of those DE gene sets indicated that those DE gene sets were highly consistent. The mean reproducibility ranged from 89.38% at *FDR* < 0.1 to 98.90% at *FDR* < 0.001 (Figure 2). Even the lowest reproducibility (71.74%) observed in the comparison of GSE42568 with GSE5764 at *FDR* < 0.1 was

unlikely to happen by chance (*P* < 0.0023). High reproducibility of the DE gene sets suggested that integrating those DE gene sets could identify the common gene expression changes of the bulk BC tissues.

To control the number of false positive DE genes and simultaneously retain an adequate number of DE genes for following analysis, we selected the genes dysregulated (*FDR* < 0.001) in at least four datasets with the same dysregulation direction and, at the same time, these selected genes did not dysregulate in the opposite direction in the remaining dataset. Nine

A cytokine predictor for side effects of tamoxifen

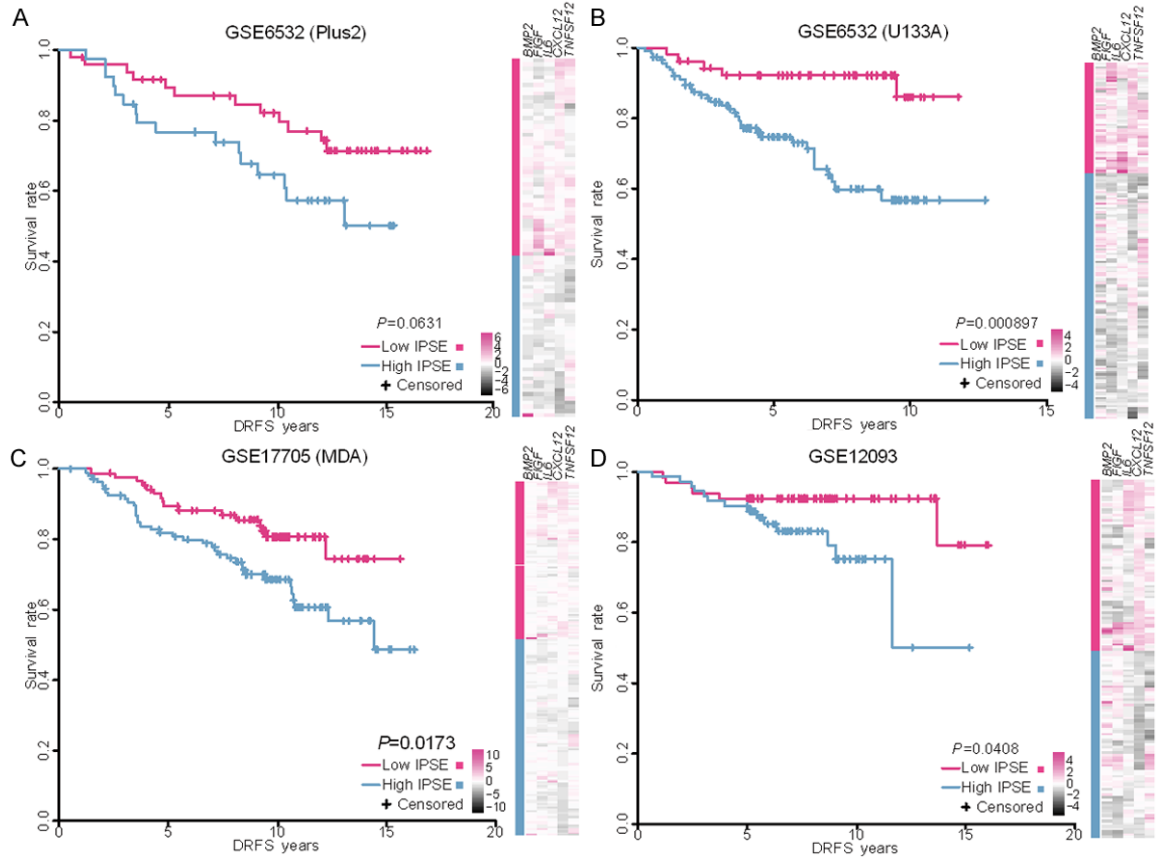


Figure 4. Kaplan-Meier survival curves for DRFS of tamoxifen-treated patients. Patients receiving tamoxifen monotherapy were stratified into the low or high IPSE group based on gene expression of the five cytokines in sub-datasets GSE6532 (Plus2) (A), GSE6532 (U133A) (B), GSE17705 (MDA) (C) and GSE12093 (D). Heatmap indicates the expression levels of the five cytokines. *P* values were calculated by log-rank tests. IPSE, immune-polarizing side effects.

hundred and ninety-five dysregulated genes met this criterion and were considered as the BC DEGs ([Online Table 3](#)).

The predicted IPSE status correlated with the DRFS of tamoxifen-treated patients

Certain cytokines may inhibit T cell function [30], preferentially attracting polarized Th2 cells [33] or polarizing immunity in the Th2 direction [34, 35]. Here, we selected DE cytokines observed in the bulk BC tissues (BC DEGs) with at least one of the corresponding receptors dysregulated in the intratumoral CD4⁺ T cells (CD4 DEGs, *FDR* < 0.001) to mirror the Th2 polarization status of CD4⁺ T cells. The selected cytokines are bone morphogenetic protein 2 (*BMP2*, Entrez gene ID: 650), c-fos induced growth factor (*FIGF*, Entrez gene ID: 2277), interleukin 6 (*IL6*, Entrez gene ID: 3569), chemokine (C-X-C motif) ligand 12 (*CXCL12*, Entrez gene ID: 6387) and tumor necrosis fac-

tor (ligand) superfamily, member 12 (*TNFSF12*, Entrez gene ID: 8742). Patients in the validation cohort were divided into the low or high Th2 polarization groups based on the expression levels of those five cytokines. As previous studies have shown that tamoxifen may cause similar immune modulation of CD4⁺ T cells as observed in BC microenvironment [14-16], we anticipated that a lower Th2 polarization before tamoxifen treatment might help counteract the CD4⁺ T cell-polarizing effects of tamoxifen [17]. Based on this assumption, the low Th2 groups should correspond to the low IPSE groups. Since *IL4* and *IL13* are Th2-type cytokines [12], their expression levels should reflect the extent of Th2 immunity within the BC microenvironment. We examined the transcriptional differences of *IL4* and *IL13* between the high and the low IPSE (Th2 polarization) groups. The expression levels of both *IL4* and *IL13* were significantly higher in the high IPSE groups than in the low IPSE groups in GSE6532 (U133A) and

A cytokine predictor for side effects of tamoxifen

Table 4. Univariate and multivariate analysis of correlation with DRFS in validation cohort

Datasets	Parameters	Univariate analysis		Multivariate analysis	
		P	HR (95% CI)	P	HR (95% CI)
GSE6532 (U133A)	Age (≥ 65 vs. < 65)	0.5515	1.209 (0.6473-2.256)	-	-
	Grade (3 vs. 1 or 2)	0.03264	2.178 (1.047-4.529)	0.1509	1.719 (0.8208-3.600)
	Size (> 2 cm vs. ≤ 2 cm)	0.002265	3.148 (1.448-6.844)	0.0502	2.332 (0.994-5.443)
	Nodal status (positive vs. negative)	0.006078	2.455 (1.265-4.766)	0.0552	2.074 (0.9839-4373)
	Predicted IPSE (low vs. high)	0.000897	4.326 (1.688-11.08)	0.0482	2.249 (1.0063-5.027)
GSE17705 (MDA)	Nodal status (positive vs. negative)	0.0001722	2.881 (1.616-5.135)	0.000567	2.7689 (1.552-4.941)
	Predicted IPSE (low vs. high)	0.0173	2.014 (1.118-3.626)	0.0448	1.864 (1.015-3.425)
GSE12093	Predicted IPSE (low vs. high)	0.04077	2.72 (1.007-7.347)	-	-

IPSE, immune-polarizing side effects.

GSE12093 (Figure 3). For patients with high IPSE in GSE17705 (MDA), only the expression level of *IL4* was significantly higher. Notably, the gene expression of both *IL4* and *IL13* in the high IPSE groups was tended to exceed those of the low IPSE groups in all sub-datasets.

Patients with low IPSE would have better prognoses after tumor removal. We looked into their possible correlation between the predicted IPSE status and the distant relapse risk of tamoxifen-treated patients. For GSE6532 (Plus2), a marginal correlation was observed (Figure 4A). Low IPSE could predict better DRFS in sub-datasets GSE6532 (U133A), GSE17705 (MDA) and GSE12093 (Figure 4B-D). Then, we applied multivariate analyses on GSE6532 (U133A) and GSE17705 (MDA) where other clinical parameters were available and also significantly correlated with patients' survival. We found that only the predicted IPSE status ($HR = 2.249$, $95\% CI 1.0063-5.027$, $P = 0.0482$) remained to be an independent prognostic factor for DRFS in GSE6532 (U133A) (Table 4). Similarly, in GSE17705 (MDA), nodal status ($HR = 2.7689$, $95\% CI 1.552-4.941$, $P = 0.000567$) and IPSE status ($HR = 1.8640$, $95\% CI 1.015-3.425$, $P = 0.0448$) were both delineated as independent prognostic factors for DRFS (Table 4). These results were consistent with the observation in GSE12093 (Figure 4D, Table 4), where patients were all lymph node negative. Those results showed that the predicted IPSE status could be used as a potential predictor for tamoxifen treatment.

The predicted IPSE status could predict the benefit of tamoxifen treatment in ER+ patients

On one hand, tamoxifen could inhibit tumor progression though targeting ER, on the other hand, the usage of tamoxifen could undermine

the anti-tumor immunity within the tumor micro-environment. So, the benefit of tamoxifen treatment among patients with different predicted IPSE status should be assessed. We analyzed the differences in DRFS between patients treated with tamoxifen and those receiving no systematic therapy in GSE6532 (U133A) and GSE6532 (U133A untreated: ER+ patients, $n = 86$). The tamoxifen-treated patients with low IPSE had significant improvement of DRFS compared with their untreated counterparts ($HR = 2.908$, $95\% CI 1.006-8.406$, $P = 0.03-905$, Figure 5). Conversely, for the patients with high IPSE, no significant DRFS improvement was observed ($P = 0.827$, Figure 5). This finding indicated that the CD4+ T cell polarizing effects of tamoxifen significantly limit the anti-tumor efficacy of this anti-estrogen agent. Therefore, the predicted IPSE status that we report here might be used as an indicator to guide the use of tamoxifen.

Discussion

In this study, we identified a Th2 biased gene expression of the intratumoral CD4+ T cells, which has previously been shown to suppress the anti-tumor immunity of the host [10-12, 33]. By integrating the DE genes of the intratumoral CD4+ T cells (CD4 DEGs) with the DE genes of the bulk BC tissues (BC DEGs), we identified five dysregulated cytokines as the predictor to reflect the Th2 polarization of the intratumoral CD4+ T cells, which in turn could be used to predict the immune-polarization side effects of tamoxifen treatment. Culturing the CD4+ T cells for 24 hours without the tumor microenvironment led to gene expression changes, which were highly consistent with the CD4 DEGs. This result suggested that some of the ectopic gene expression of the intratumoral

A cytokine predictor for side effects of tamoxifen

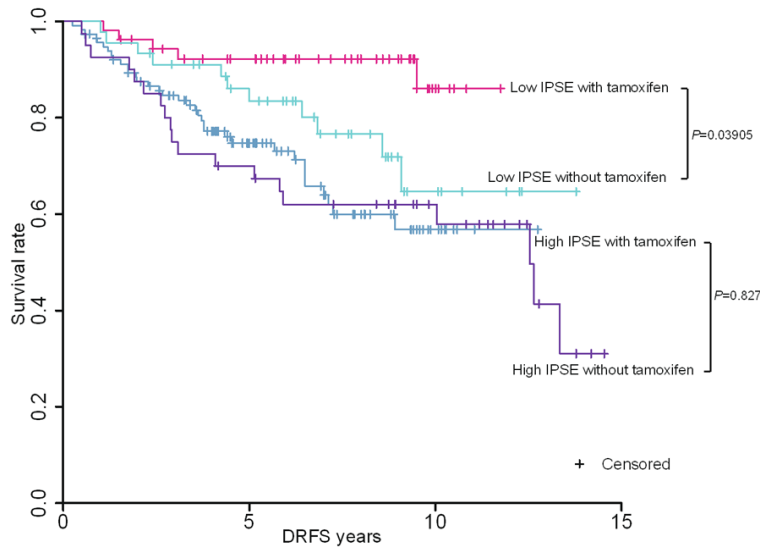


Figure 5. Kaplan-Meier survival curves for DRFS of patients with or without tamoxifen treatment. Patients receiving tamoxifen mono-adjuvant therapy and patients without any systematic treatment were stratified into the low or high IPSE group based on gene expression of five cytokines. Differences in DRFS of patients of the same SE status were assessed. SE, side effect.

CD4+ T cells might be transient. Thus, reversing the malfunction of the intratumoral CD4+ T cell through modulating the levels of cytokines might improve the prognoses of patients with high IPSE of tamoxifen. Our results also suggest that only patients predicted with the low IPSE might benefit from the tamoxifen treatment, indicating that the CD4+ T cell modulating effect of tamoxifen on the intratumoral CD4+ T cells might be a pivotal step in hindering the anti-tumor effect of tamoxifen treatment [17]. The combined expression levels of five cytokines could be a potential molecular guidance to help determine whether a BC patient should receive a tamoxifen therapy.

The five cytokines are involved in different biological aspects of the CD4+ T cells. BMP2 can modulate T cell proliferation, and functional BMP receptors have been detected in peripheral murine CD4+ T lymphocytes [36]. The BMP receptors are expressed mainly in activated T cells and the BMP signaling is a physiological component of CD4+ T cell responses [36]. IL-6, another multifunctional factor to CD4+ T cells, can prevent T cells from entering apoptosis [37, 38]. We also found enhanced apoptosis of CD4+ T cells within tumor microenvironment (Online Table 1), possibly due to the inhibited function of IL-6. Some groups have found that IL-6 affects B cell helper capabilities of CD4+ T cells through elevating IL-21 production [39,

40]. CXCL12, also known as stromal cell derived factor (SDF) 1, is a co-stimulator for peripheral CD4+ T cells and enhances the activation of marker expression, proliferation, and cytokine production by anti-CD3-stimulated peripheral CD4+ T cells [41]. CXCL12 alone could stimulate CD4+ memory T cells and has been found to co-stimulate responses of anti-CD3-stimulated CD4+ naïve and memory T cells as well as CD8+ T cells [42]. Cytokines work in synergy. The mechanisms of the combined cytokines influencing the functionality of CD4+ T cells remained to be determined.

In the renaissance of interests in the tumor microenvironment, not much attention has been paid to the stroma-induced drug resistance or sensitization [1]. Traditional studies focusing on biomarkers for tamoxifen sensitivity [43, 44] were usually with ambiguous biological interpretation, which may provide limited strategies to improve the drug efficacy. Our study aims at a new aspect of IPSE of tamoxifen on the stromal components, the CD4+ T cells, which might lead to novel insights into the mechanisms of immune malfunction within the tumor microenvironment and strategies to improve the effect of tamoxifen treatment.

Current approaches to immunotherapy usually achieve limited effects partly due to the immune suppression taking place within the solid tumor [45]. Some immune therapies were the paradigm that inducing more antitumor immune cells alone. Based on the immune shift of CD4+ T cells observed within solid tumors, we propose that those strategies should be revised. Our study demonstrated that therapeutics targeting the CD4+ T cells should be used before or in combination with the tamoxifen therapy to improve the benefit or reduce the IPSE of tamoxifen therapy.

Acknowledgements

This work was supported by grants of the National Natural Science Foundation of China

(NSFC30970119, 81030029, 81271786, NS-FC-NIH 81161120416). No medical writers were involved in writing this manuscript.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Shu-Lin Liu, Genomics Research Center (one of The State-Province Key Laboratories of Biomedicine-Pharmaceutics of China), Harbin Medical University, China. Tel: +8645186614075; Fax: +8645186614075; E-mail: slliu@ucalgary.ca

References

- [1] McMillin DW, Negri JM and Mitsiades CS. The role of tumour-stromal interactions in modifying drug response: challenges and opportunities. *Nature reviews. Nat Rev Drug Discov* 2013; 12: 217-228.
- [2] Hanahan D and Weinberg RA. Hallmarks of cancer: The next generation. *Cell* 2011; 144: 646-674.
- [3] Coussens LM and Werb Z. Inflammation and cancer. *Nature* 2002; 420: 860-867.
- [4] Georgiannos SN, Renaut A, Goode AW and Sheaff M. The immunophenotype and activation status of the lymphocytic infiltrate in human breast cancers, the role of the major histocompatibility complex in cell-mediated immune mechanisms, and their association with prognostic indicators. *Surgery* 2003; 134: 827-834.
- [5] Matkowski R, Gisterek I, Halon A, Lacko A, Szweczyk K, Staszek U, Pudelko M, Szynglarewicz B, Szelachowska J, Zolnierok A and Kornafel J. The prognostic role of tumor-infiltrating CD4 and CD8 T lymphocytes in breast cancer. *Anti-cancer Res* 2009; 29: 2445-2451.
- [6] Zitvogel L, Tesniere A and Kroemer G. Cancer despite immunosurveillance: immunoselection and immunosubversion. *Nature reviews. Immunology* 2006; 6: 715-727.
- [7] Dobrzanski MJ. Expanding roles for CD4 T cells and their subpopulations in tumor immunity and therapy. *Front Oncol* 2013; 3: 63.
- [8] Bos R and Sherman LA. CD4+ T-cell help in the tumor milieu is required for recruitment and cytolytic function of CD8+ T lymphocytes. *Cancer Res* 2010; 70: 8368-8377.
- [9] Disis ML. Immune regulation of cancer. *J Clin Oncol* 2010; 28: 4531-4538.
- [10] Ruffell B, Au A, Rugo HS, Esserman LJ, Hwang ES and Coussens LM. Leukocyte composition of human breast cancer. *Proc Natl Acad Sci U S A* 2012; 109: 2796-2801.
- [11] Pedroza-Gonzalez A and Xu K. Thymic stromal lymphopoietin fosters human breast tumor growth by promoting type 2 inflammation. *J Exp Med* 2011; 208: 479-490.
- [12] DeNardo DG, Barreto JB, Andreu P, Vasquez L, Tawfik D, Kolhatkar N and Coussens LM. CD4(+) T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages. *Cancer Cell* 2009; 16: 91-102.
- [13] Aspod C, Pedroza-Gonzalez A, Gallegos M, Tindle S, Burton EC, Su D, Marches F, Banchereau J and Palucka AK. Breast cancer instructs dendritic cells to prime interleukin 13-secreting CD4+ T cells that facilitate tumor development. *J Exp Med* 2007; 204: 1037-1047.
- [14] Robinson E, Rubin D, Mekori T, Segal R and Pollack S. In vivo modulation of natural killer cell activity by tamoxifen in patients with bilateral primary breast cancer. *Cancer Immunol Immunother* 1993; 37: 209-212.
- [15] Bebo BF, Dehghani B, Foster S, Kurniawan A, Lopez FJ and Sherman LS. Treatment with selective estrogen receptor modulators regulates myelin specific T-cells and suppresses experimental autoimmune encephalomyelitis. *Glia* 2009; 57: 777-790.
- [16] de Kozak Y, Andrieux K, Villarrojo H, Klein C, Thillaye-Goldenberg B, Naud MC, Garcia E and Couvreur P. Intraocular injection of tamoxifen-loaded nanoparticles: a new treatment of experimental autoimmune uveoretinitis. *Eur J Immunol* 2004; 34: 3702-3712.
- [17] Behjati S and Frank MH. The effects of tamoxifen on immunity. *Curr Med Chem.* 2009; 16: 3076-3080.
- [18] Frank MH, Denton MD, Alexander SI, Khoury SJ, Sayegh MH and Briscoe DM. Specific MDR1 P-glycoprotein blockade inhibits human alloimmune T cell activation in vitro. *J Immunol* 2001; 166: 2451-2459.
- [19] Pendse SS, Behjati S, Schatton T, Izawa A, Sayegh MH and Frank MH. P-glycoprotein functions as a differentiation switch in antigen presenting cell maturation. *Am J Transplant* 2006; 6: 2884-2893.
- [20] Gu-Trantien C, Loi S, Garaud S, Equeter C, Libin M, de Wind A, Ravoet M, Le Buanec H, Sibille C, Manfouo-Foutsop G, Veys I, Haibe-Kains B, Singhal SK, Michiels S, Rothé F, Salgado R, Duvillier H, Ignatiadis M, Desmedt C, Bron D, Larsimont D, Piccart M, Sotiriou C and Willard-Gallo K. CD4+ follicular helper T cell infiltration predicts breast cancer survival. *J Clin Invest* 2013; 123: 2873-2892.
- [21] Bolstad BM, Irizarry Ra, Astrand M and Speed TP. A comparison of normalization methods for

A cytokine predictor for side effects of tamoxifen

- high density oligonucleotide array data based on variance and bias. *Bioinformatics* 2003; 19: 185-193.
- [22] Tusher VG, Tibshirani R and Chu G. Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci U S A* 2001; 98: 5116-5121.
- [23] Zhang S. A comprehensive evaluation of SAM, the SAM R-package and a simple modification to improve its performance. *BMC Bioinformatics* 2007; 8: 230.
- [24] Huang DW, Sherman BT and Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res* 2009; 37: 1-13.
- [25] Huang DW, Sherman BT and Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 2009; 4: 44-57.
- [26] Zhang M, Yao C, Guo Z, Zou J, Zhang L, Xiao H, Wang D, Yang D, Gong X, Zhu J, Li Y and Li X. Apparently low reproducibility of true differential expression discoveries in microarray studies. *Bioinformatics* 2008; 24: 2057-2063.
- [27] Zhang M, Zhang L, Zou J, Yao C, Xiao H, Liu Q, Wang J, Wang D, Wang C and Guo Z. Evaluating reproducibility of differential expression discoveries in microarray studies by considering correlated molecular changes. *Bioinformatics* 2009; 25: 1662-1668.
- [28] Zhou X, Shi T, Li B, Zhang Y, Shen X, Li H, Hong G, Liu C and Guo Z. Genes dysregulated to different extent or oppositely in estrogen receptor-positive and estrogen receptor-negative breast cancers. *PLoS One* 2013; 8: e70017.
- [29] Mantovani A, Allavena P, Sica A and Balkwill F. Cancer-related inflammation. *Nature* 2008; 454: 436-444.
- [30] Gajewski TF, Meng Y, Blank C, Brown I, Kacha A, Kline J and Harlin H. Immune resistance orchestrated by the tumor microenvironment. *Immunol Rev* 2006; 213: 131-145.
- [31] Cleator SJ, Powles TJ, Dexter T, Fulford L, Mackay A, Smith IE, Valgeirsson H, Ashworth A and Dowsett M. The effect of the stromal component of breast tumours on prediction of clinical outcome using gene expression microarray analysis. *Breast Cancer Res* 2006; 8: R32.
- [32] Rody A, Holtrich U, Pusztai L, Liedtke C, Gaetje R, Ruckhaeberle E, Solbach C, Hanker L, Ahr A, Metzler D, Engels K, Karn T and Kaufmann M. T-cell metagene predicts a favorable prognosis in estrogen receptor-negative and HER2-positive breast cancers. *Breast Cancer Res* 2009; 11: R15.
- [33] Balkwill F and Mantovani A. Inflammation and cancer: back to Virchow? *Lancet* 2001; 357: 539-545.
- [34] Gu L, Tseng S, Horner RM, Tam C, Loda M and Rollins BJ. Control of TH2 polarization by the chemokine monocyte chemoattractant protein-1. *Nature* 2000; 404: 407-411.
- [35] Sozzani S, Luini W, Bianchi G, Allavena P, Wells TN, Napolitano M, Bernardini G, Vecchi A, D'Ambrosio D, Mazzeo D, Sinigaglia F, Santoni A, Maggi E, Romagnani S and Mantovani A. The viral chemokine macrophage inflammatory protein-1 α is a selective Th2 chemoattractant. *Blood* 1998; 92: 4036-4039.
- [36] Yoshioka Y, Ono M, Osaki M, Konishi I and Sakaguchi S. Role of BMP signalling in peripheral CD4 $^{+}$ T cell proliferation. *Eur J Immunol* 2012; 42: 749-759.
- [37] Atreya R, Mudter J, Finotto S, Müllberg J, Jostock T, Wirtz S, Schütz M, Bartsch B, Holtmann M, Becker C, Strand D, Czaja J, Schlaak JF, Lehr HA, Autschbach F, Schürmann G, Nishimoto N, Yoshizaki K, Ito H, Kishimoto T, Galle PR, Rose-John S and Neurath MF. Blockade of interleukin 6 trans signaling suppresses T-cell resistance against apoptosis in chronic intestinal inflammation: evidence in crohn disease and experimental colitis in vivo. *Nat Med* 2000; 6: 583-588.
- [38] Jones SA. Directing transition from innate to acquired immunity: defining a role for IL-6. *J Immunol* 2005; 175: 3463-3468.
- [39] Dienz O, Eaton SM, Bond JP, Neveu W, Moquin D, Noubade R, Briso EM, Charland C, Leonard WJ, Ciliberto G, Teuscher C, Haynes L and Rincon M. The induction of antibody production by IL-6 is indirectly mediated by IL-21 produced by CD4 $^{+}$ T cells. *J Exp Med* 2009; 206: 69-78.
- [40] Eddahri F, Denanglaire S, Bureau F, Spolski R, Leonard WJ, Leo O and Andris F. Interleukin-6/STAT3 signaling regulates the ability of naive T cells to acquire B-cell help capacities. *Blood* 2009; 113: 2426-2433.
- [41] Nanki T and Lipsky P. Cutting edge: stromal cell-derived factor-1 is a costimulator for CD4 $^{+}$ T cell activation. *J Immunol* 2000; 164: 5010-5014.
- [42] Nanki T and Lipsky P. Stimulation of T-Cell activation by CXCL12/stromal cell derived factor-1 involves a G-protein mediated signaling pathway. *Cell Immunol* 2001; 214: 145-154.
- [43] Zhang Y, Sieuwerts AM, McGreevy M, Casey G, Cufer T, Paradiso A, Harbeck N, Span PN, Hicks DG, Crowe J, Tubbs RR, Budd GT, Lyons J, Sweep FCGJ, Schmitt M, Schittulli F, Golouh R, Talantov D, Wang Y and Foekens JA. The 76-gene signature defines high-risk patients that benefit from adjuvant tamoxifen therapy. *Breast Cancer Res Treat* 2009; 116: 303-309.
- [44] Symmans WF, Hatzis C, Sotiriou C, Andre F, Peintinger F, Regitnig P, Daxenbichler G, Desmedt C, Domont J, Marth C, Delaloge S, Bauernhofer T, Valero V, Booser DJ, Hortobagyi GN and Pusztai L. Genomic index of sensitivity to endocrine therapy for breast cancer. *J Clin Oncol* 2010; 28: 4111-4119.
- [45] Curiel TJ. Tregs and rethinking cancer immunotherapy. *J Clin Invest* 2007; 117: 1167-1174.