Original Article

Identification of potential therapeutic targets using breast cancer stroma expression profiling

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Background: To uncover the potential molecular mechanisms of cytokine-relevant genes in breast cancer stroma and identify potential targets for breast cancer treatment.

Methods: The *differentially expressed* (*DE*) *cytokine* genes in breast cancer stroma were assessed using microarray data. The pathway and functional enrichment analyses were performed. A protein-protein interaction (PPI) network and PPI subnetworks were constructed and the subnetwork was analyzed by Cytoscape software.

Results: One hundred and three *DE cytokine* genes (55 up-regulated and 48 down-regulated) were identified. Functional enrichment and pathway analysis showed that inflammation, blood vessel growth, leuko-monocyte differentiation, extracellular matrix (ECM) turnover and remodeling pathways were involved. The PPI network with 85 nodes and 236 interactions was constructed and three subnetworks were also identified. Genes encoding for CXCL12, TLR2, ITGAM, and SOCS3 were extracted as the hub genes.

Conclusions: These dysregulated genes of tumor stroma may provide important clues for in-depth study of breast cancer therapeutic strategies.

Keywords: Breast cancer; cytokine; stroma; therapeutic targets

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Introduction

Breast cancer is a major cause of morbidity and mortality in women worldwide (1). Breast cancer is recognized as a heterogeneous disease. Even when morphological characteristics and ER phenotypes are similar, patients have varying prognosis or chemotherapy response (2). Therefore, individual treatment for breast cancer patients requires modification to a more practical clinical signature.

The view of cancer as a systemic disease indicates that the development of malignant tumors is involved in a complex biological process that be directed by the body's systemic responses to malignancy (3). Paraneoplastic syndromes are the manifestation of systemic complications caused by organ or tissue damage at locations remote from the site of the primary tumor or metastases (4), such as leukocytosis and thrombocytosis, commonly associated multiple type of malignancies including breast cancer (5). Primary tumors communicate with distant tissues by varied means, such as releasing of soluble cytokines into the circulation. For instance, increased hepatic thrombopoietin synthesis in response to tumor-derived interleukin-6 was an underlying mechanism of paraneoplastic thrombocytosis (6). Excessive IL-6 has been demonstrated in primary breast tumors and breast cancer patient sera and is associated with poor clinical outcomes

Table 1 Clinical characteristics of patients (n=53)

Category	Patients
Mean age at operation (years)	51.4
HER2 -positive	10
ER -positive	43
PR -positive	27
Lymph node positive	25
Pathological stage	
I	17
IIA	19
IIB	10
IIIA	2
IIIC	2
NA	3
Grade	
I	3
II	23
III	27

in breast cancer (7,8).

Carcinomas are composed of malignant epithelial cells and complex tissue environment of diverse stromal cells, which they depend upon for sustained growth, invasion and metastasis. In addition to tumor-driven systemic perturbations, the stroma cells of tumor microenvironment (TME) be educated and sculpted by tumor cells via paracrine and juxtacrine, may also have capacity of promoting systemic effects (9).

Cytokines and chemokines are important constituents of the TME. Increasing number of studies indicate that cytokines and chemokines function as critical regulators involving immune cell responses, angiogenesis, lymphangiogenesis, extracellular matrix (ECM) dynamics (10-12). These studies implied that cytokines and chemokines may be commonly used in para-tumor context. Herein, we hypothesized that the cytokine expression pattern is potentially a manifestation of paraneoplastic responses, which seems to be consistent in breast cancer patients. Further specification of this cytokine pattern could offer novel therapeutic approaches.

Methods

Data sources

Microarray data, accession number GSE9014, deposited by Finak *et al.* (13), were downloaded from gene expression omnibus (GEO) (14), samples included 53 cases (50 invasive ductal carcinoma IDC and 3 invasive lobular carcinoma cases) of tumor stroma, and 31 cases of individual-matched normal adjacent stroma. Patient's surgical excision were frozen in liquid nitrogen within 30 min of surgical resection. A clinical pathologist identified distinct regions of tumor stroma and stroma surrounding morphologically normal ducts prior to laser-capture microdissection (LCM) (13). The clinical characteristics of the patients are showed in *Table 1* (14). All of the patients had not underwent neoadjuvant therapy before surgical (13). Genes whose expression varied most between tumor tissue and normal stroma for the 31 tissue-matched pairs of this data had been identified to be independent of ER, HER2 and lymph node status, as well as age, grade and tumor size (13). In Finak's study, all patients provided written, informed consent and the experiments were approved by the McGill University Health Centre (MUHC) Research Ethics Board (Protocols SUR-99-780 and SUR-00-966) (13).

Data preprocessing and identification of differentially-expressed genes (DEGs)

The raw data were analyzed using the integrated software package biometric research branch (BRB) ArrayTools version 4.0 (15). BRB-Array tools performed a series of preprocessing steps including filtering and computing hybridization data of each probe in the probe set. Probes with the following conditions were removed: (I) greater than 20% of expression data values had more than 1.5-fold change in either direction from the median value; (II) more than 50% of the gene expression data were "absent". The Robust Multiarray Average (RMA) method (16) was used for normalization. The statistical analysis of microarray (SAM) method (17) was used to analyze the transcription profiles and screen for DEGs [false discovery rate (FDR) ≤1%, |log (fold change, FC)|≥1].

Functional enrichment analysis of DEGs

Gene ontology (GO) (18) enrichment analysis was carried

out to uncover the biological processes of the DEGs. Additionally, the biological pathway enrichment analysis of DEGs was performed based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (19). The online based tool of the database for annotation, visualization and integrated discovery (DAVID) (20) was used to perform these analyses.

Screening of DEGs for cytokines and cytokine receptors

The cytokines, cytokine receptors, and chemokines were screened based on KEGG ko04052: Cytokines (21) and their functions are listed in NCBI Mesh (22). KEGG ko04052 collected 230 cytokines in 8 families, including class I cytokines (hematopoietic family), class II cytokines (interferon/IL-10 family), PDGF family, TNF family, IL-1 family, IL-17 family, TGF-beta family, and chemokines.

Protein-protein interaction (PPI) network construction and subnetwork analysis

PPI network of the DE cytokine and chemokine genes was established based on the Search Tool for the Retrieval of Interacting Genes (STRING) database (23), and only interactions with combined score >0.4 were selected to construct the PPI network and visualized. The property of the network was evaluated using the network analyzer plugin of Cytoscape (24). The proteins in the network served as nodes and the degree of a node corresponded to the number of interactions with other proteins. The proteins with high degrees were considered as the hub nodes. In addition, we further performed subnetwork mining in the PPI network based on KMEANS clustering algorithm to cluster the proteins displaying in the network. The only input to the clustering algorithm was the distance matrix obtained from the String global scores (therefore interacting proteins with a higher global score had more chances to end up in the same cluster).

Results

DEGs between breast cancer stroma samples and normal samples

A total of 3,420 transcripts, corresponding to 2,737 DEGs, including 1,122 upregulated DEGs and 1,615 down regulated DEGs were identified between the breast cancer stroma samples and the controls. Among these DEGs, 55 were up-regulated and 48 were down-regulated cytokines and cytokine–related genes (for instance cytokine receptors).

Functional enrichment analysis of up- and down-regulated cytokines

GO biological processes enrichment analysis revealed that 185 functional terms for DE cytokine genes (both down- and up-regulated cytokine genes) were enriched; the top five significantly enriched processes were mainly related to immune response and leukocyte activation; beyond that, the DE cytokine genes were also involved in a variety of biological processes, such as fibroblast growth factor receptor signaling pathway and angiogenesis. The GO functional terms ranked by statistical significance were listed in *Table 2*.

The up-regulated cytokines were enriched in seven pathways, which were mainly involved in pathways related to cancer such as cytokine-cytokine receptor interaction pathways and hematopoietic cell lineage. Meanwhile, the down-regulated cytokines were enriched in four pathways, which were cytokine-cytokine receptor interaction, toll-like receptor (TLR) signaling pathway, chemokine signaling pathway, cell adhesion molecules (CAMs). The significantly enriched pathways of up- and down-regulated cytokines are listed in *Table 3*.

PPI network construction and subnetwork analysis

The PPI network of DE cytokine genes (both down- and up-regulated) was constructed, and included 85 nodes and 236 interactions, as shown in *Figure 1*. Nodes with large degree and high BC (Betweenness Centrality, a metric that captures the importance of each individual node in the overall network structure) are represented as the hub genes displayed in *Table 4*. Furthermore, three subnetworks were selected from DE PPI pairs, *Figure 2*. The hub proteins CXCL12, CXCR4, KIT, TIMP1, and CD34 were demonstrated to be involved in subnetwork 1, while TLR2, ITGAM, and CD36 were in subnetwork 2, and SOCS3 in subnetwork 3.

Discussion

During tumor progression, a variety of factors released by tumor tissue not only regulate the local microenvironment, but also regulate distal tissues. Alterations within the tumor macro-environment, leads to the promotion of

Table 2 Significantly enriched GO biological processes for differentially-expressed cytokine genes

Term	Count	P value
Immune response	25	9.04E-12
Cell surface receptor linked signal transduction	38	8.80E-11
Chemotaxis	13	5.36E-10
Leukocyte activation	15	5.48E-10
Defense response	21	2.18E-09
Cell adhesion	22	3.40E-09
Integrin-mediated signaling pathway	9	1.72E-08
Inflammatory response	15	2.43E-08
Response to wounding	18	5.17E-08
Regulation of cell proliferation	20	6.49E-07
Positive regulation of immune system process	11	3.54E-06
Regulation of programmed cell death	19	4.52E-06
Lymphocyte activation	10	6.20E-06
Cell migration	11	1.31E-05
T cell activation	8	1.88E-05
Protein kinase cascade	12	2.98E-05
Cell motility	11	3.26E-05
Fibroblast growth factor receptor signaling pathway	5	3.67E-05
Positive regulation of cell communication	11	5.85E-05
Enzyme linked receptor protein signaling pathway	11	8.08E-05
Cytokine-mediated signaling pathway	6	9.51E-05
Transmembrane receptor protein tyrosine kinase signaling pathway	9	1.11E-04
Regulation of cytokine production	8	1.87E-04
Myeloid leukocyte activation	5	2.31E-04
Innate immune response	7	2.93E-04
Hematopoietic or lymphoid organ development	9	3.08E-04
Positive regulation of cell migration	6	2.96E-04
Angiogenesis	7	4.27E-04
Cell-substrate adhesion	6	4.62E-04

Counts, number of genes.

Table 3 Significantly enriched KEGG pathways for differentially-expressed cytokine genes

Term	Count	P value
Up-regulated pathways		
Cytokine-cytokine receptor interaction	17	5.84E-14
Hematopoietic cell lineage	9	1.55E-08
Regulation of actin cytoskeleton	7	0.001117
JAK-STAT signaling pathway	6	0.002207
Chemokine signaling pathway	5	0.020481
Intestinal immune network for IgA production	3	0.031302
Pathways in cancer	6	0.046081
Down-regulated pathways		
Cytokine-cytokine receptor interaction	10	3.22E-06
Toll-like receptor signaling pathway	6	1.32E-04
Chemokine signaling pathway	6	0.002233
Cell adhesion molecules (CAMs)	4	0.028752

Counts, number of genes.

tumor growth, invasion, and eventual metastasis (25). Though tumor cell is master regulator of the tumor macro-environment, other types of cells in tumor microenvironment may also coordinate the development of cancer-associated systemic syndromes.

In this study, using mRNA expression profile, we focused on breast cancer stroma, and identified 56 up-regulated and 48 down-regulated genes of cytokines or chemokines. GO functional annotation and KEGG pathway analyses revealed that the DE cytokines were enriched in a variety of biological progresses (BP). These multiple progresses were in relation to inducing inflammation, blood vessel growth, leuko-monocyte differentiation, ECM turnover and remodeling, thus reflecting the continual changes and evolution of the tumor macro- and micro-environment as the tumor grows.

PPIs analysis can visualize functional links between DEGs at the molecular level, and is useful in helping understand the molecular mechanism of diseases and their potentially essential genes. We therefore constructed the PPI network between the DE cytokines, and observed three subnetworks with highly connected nodes, using K-MEANS

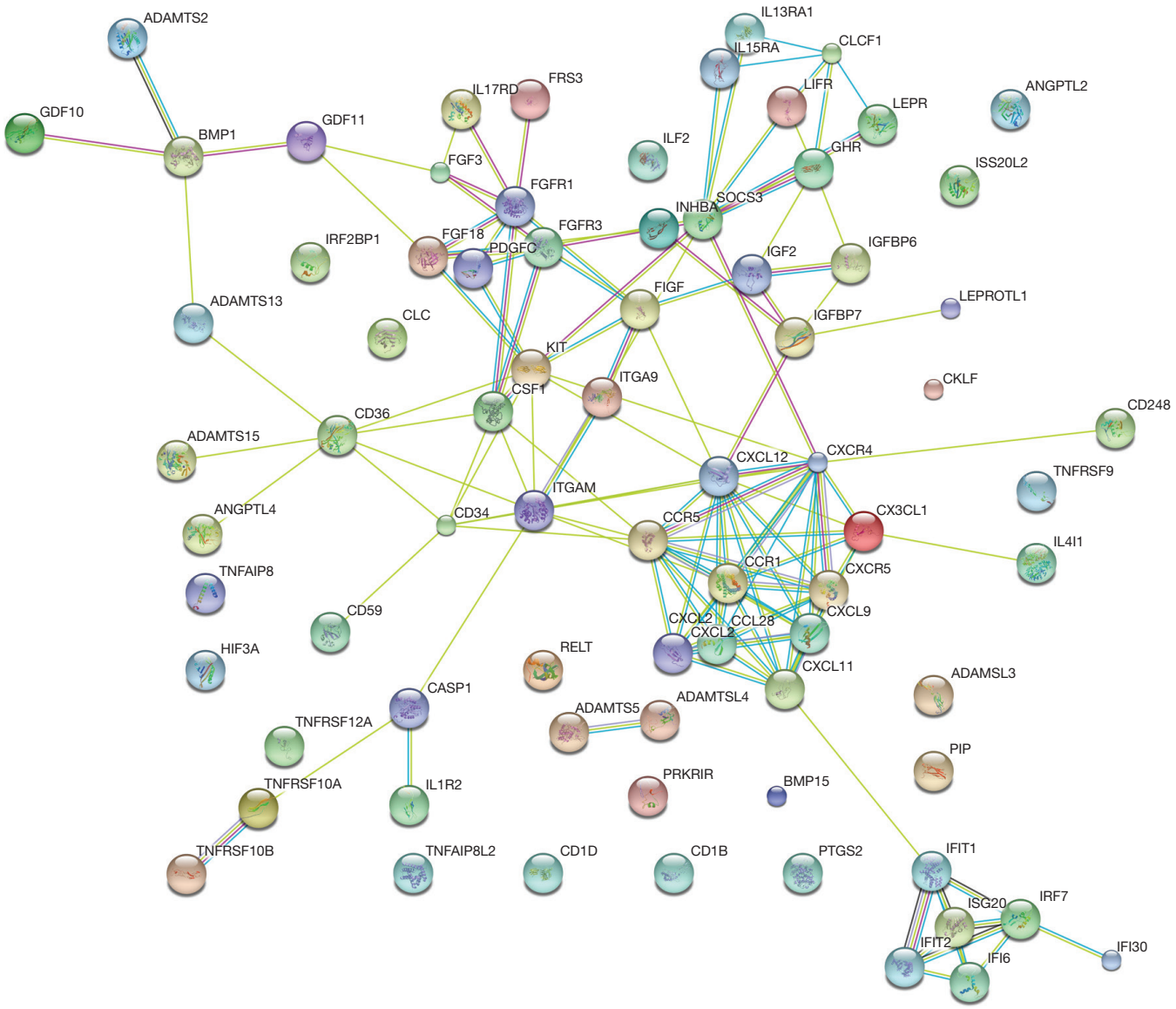

Figure 1 The network nodes are proteins. The edges represent the predicted functional associations. An edge may be drawn with up to seven differently colored lines, which represent the existence of the seven types of evidence used in predicting the associations. A red line, the presence of fusion evidence; a green line, neighborhood evidence; a blue line, co-ocurrence evidence; a purple line, experimental evidence; a yellow line, text-mining evidence; a light blue line, database evidence; a black line, co-expression evidence.

clustering algorithm.

Subnetwork 1 was mainly enriched in two KEGG pathways, cytokine-cytokine receptor interaction and chemokine signaling pathway. CXCL12 and its receptor CXCR4, were the hub proteins of subnetwork 1. CXCL12, also known as stromal-derived factor 1α (SDF-1), is a secreted protein expressed widely in immune cells, endothelial cells, stromal fibroblasts (26). Through CXCL12/CXCR4 axis, interplays exist not only between the local neighboring cells, but also between the tumor microenvironment and the patient's organs and systems. Megakaryocytes (MKs) are specialized precursor cells that produce platelets. MKs express the receptor CXCR4 in response to SDF1 and to increase their platelet production (27). A recent study shows that SDF-1 increases megakaryocyte-vascular association and resulting in

Table 4 Hub nodes sorted by degree score

Node	Degree score	BC	Dysregulation
CXCL12	21	0.139614	up
TLR2	18	0.191426	down
ITGAM	18	0.15498	up
CXCR4	16	0.063763	down
KIT	13	0.094832	up
CD36	12	0.109278	up
TIMP1	11	0.099352	down
CD34	11	0.062662	up
SOCS3	10	0.133901	up

Node degree and Betweenness Centrality (BC) are topological parameters used for gene prioritization in the network; a cut-off of BC >0.05 and/or node degree >10 were considered.

thrombopoiesis (28). Paraneoplastic thrombocytosis is associated with many solid tumors and can promote tumor growth and metastasis (29). The present study revealed that the *CXCL12* gene was up-regulated in the stroma of breast cancer and it was a hub protein with the highest degree score in the established PPI network. Therefore, CXCL12 may be a key regulator in stroma-host interactions as in breast cancer development. Potentially, therapeutic strategies targeting CXCL12 may lead to pleiotropic treatments for breast cancer by interrupting stroma-driven systemic perturbations.

ITGAM and TLR2 were the hub proteins of subnetwork 2. ITGAM (Integrin Alpha M), encoding CD11b, is the common marker of myeloid-derived suppressor cells (MDSCs) (30). CD11b binds to ligands to regulate leukocyte adhesion and migration across the endothelium to inflammatory sites (31). The expression

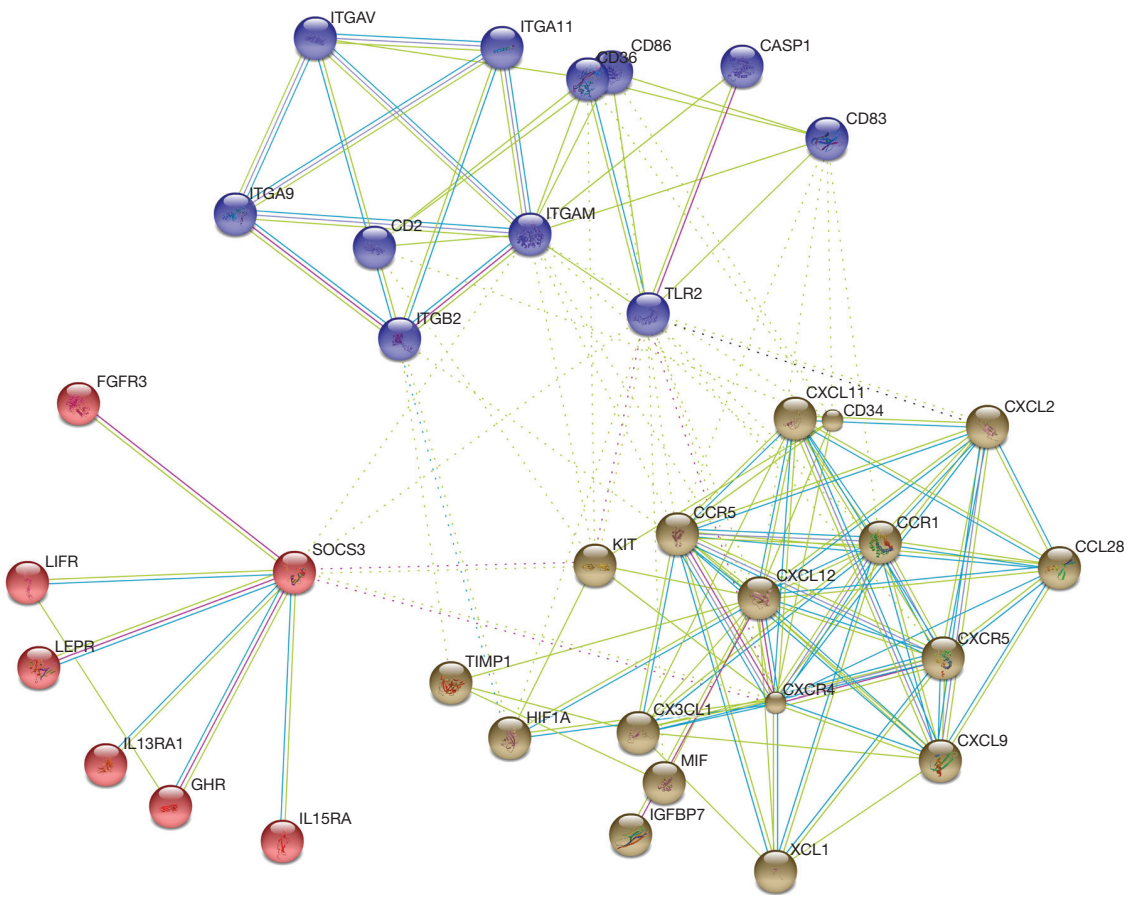

Figure 2 The three significant subnetworks in the protein-protein interaction network. The node stands for the protein (gene); every color corresponds to a cluster, subnetwork 1 is showed in brown, subnetwork 2 in blue, subnetwork 3 in red. The edges represent the predicted functional associations, as illustrated in *Figure 1*. Inter-cluster edges are represented by dashed-lines.

of ITGAM was significantly up-regulated in breast tumor stroma indicating the accumulation of MDSCs in the tumor microenvironment, which is a paraneoplastic phenomenon of cancer-associated myeloproliferation (32). The immature myeloid cells lead to the suppression of host immunity and promotion of tumor angiogenesis, both of which play a role in tumorigenesis and metastasis, representing a novel therapeutic target in cancer (33). Research in colorectal cancer (CRC) shows that CD11b deficiency contributes to the inhibition of myeloid cell trafficking to the tumor microenvironment and thereby suppresses the angiogenesis and tumor growth (34). Since CD11b collectively expressed on the surface of a variety of myeloid cells across different tumor types, it would be a potential therapeutic target in breast cancer.

TLRs are key factors in the innate immune system, which recognize antigens that are not normally expressed within the host, and initiate the inflammatory response by promoting the release of cytokines, chemokines, and other agents required in antitumor immunity (35). TLR2 is found in a large diversity of cells of the immune system. It has been implicated in the response to tumor-derived factors, and that genetic ablation of TLR2 in primary bone marrow-derived macrophages abolished the activation effect in breast cancer (36). In the present study,TLR2 was down-regulated in breast cancer stroma cells and was hub node in the same PPI subnetwork with CD11b. We supposed that both of the two molecules might play a key role in the development of MDSCs in breast cancer. Given the emerging importance of MDSCs in breast cancer, modulating MDSCs actions is an attractive avenue of further therapy research for those incurable breast cancers (37).

Suppressor of cytokine signaling protein 3 (SOCS3), hub protein of the subnetwork 3, is negative regulator of cytokine signaling mediated by the JAK-STAT signaling pathway. Many studies suggest that macrophage SOCS3 is associated with M1 macrophages and pro-inflammatory responses. However the exact consequences of SOCS3 signaling, either positive or negative, are still controversial (38-40). Heys *et al.* (41), determined that the number of SOCS3-expressing pro-inflammatory and cytotoxic macrophages correlated with early response to therapy, but not with worse survival. SOCS3 expression may identify macrophages with enhanced tumor-killing capacity. Moreover, in addition to macrophages, the function of endothelial cells and dendritic cells are affected by SOCS3 (42,43). Targeting SOCS3 may therefore be important an efficient means of tailoring the inflammatory response in tumor biology.

In conclusion, we found that tumor stroma gene expression profiles were markedly altered during the development and progression of breast cancer. Specifically, cytokines, as well as their related genes, may be important in the paraneoplastic response. We identified CXCL12, TLR2, ITGAM, and SOCS3 as the key genes of cytokine regulatory network, thus may be potential targets in the development of treatments for breast cancer. However, because the results are based on microarray data with a small sample size, more experimental validations are warranted. Further studies are necessary to evaluate their potential applications as therapeutic targets.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2016.05.07). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). In Finak's study, all patients provided written, informed consent and the experiments were approved by the McGill University Health Centre (MUHC) Research Ethics Board (Protocols SUR-99-780 and SUR-00-966).

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