Bioinformatic analysis of computational identified differentially expressed genes in tumor stoma of pregnancy-associated breast cancer

QIAN ZHOU[1*], ERHU SUN[1*], LIJUN LING[2], XIAOFENG LIU[1], MIN ZHANG[1], HONG YIN[1] and CHENG LU[1]

[1]Department of Breast, Nanjing Maternity and Child Health Care Hospital, Obstetrics and Gynecology Hospital Affiliated to Nanjing Medical University, Nanjing, Jiangsu 210004; [2]Department of Breast Surgery, The First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu 210029, P.R. China

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Abstract. The present study aimed to screen the differentially expressed genes (DEGs) in tumor-associated stroma of pregnancy-associated breast cancer (PABC). By analyzing Affymetrix microarray data (GSE31192) from the Gene Expression Omnibus database, DEGs between tumor associated stromal cells and normal stromal cells in PABC were identified. Gene Ontology (GO) function and pathway enrichment analyses for the DEGs were then performed, followed by construction of a protein-protein interaction (PPI) network. A total of 94 upregulated and 386 downregulated DEGs were identified between tumor associated stromal cells and normal stromal cells in patients with PABC. The upregulated DEGs were primarily enriched in the cytokine-cytokine receptor interaction pathway and GO terms associated with the immune response, which included the DEGs of interleukin 18 (*IL18*) and cluster of differentiation 274 (*CD274*). The downregulated DEGs were primarily involved in GO terms associated with cell surface receptor linked signal transduction and pathways of focal adhesion and pathways in cancer. In the PPI network, nodes of jun proto-oncogene (*JUN*), FBJ murine osteosarcoma viral oncogene homolog (*FOS*), V-myc avian myelocytomatosis viral oncogene homolog (*MYC*), and alpha-smooth muscle actin (*ACTA2*) had higher degrees. The hub genes of *JUN*, *FOS*, *MYC* and *ACTA2*, as well as the DEGs *IL18* and *CD274* that were associated with the immune response in GO terms may exert important functions in the molecular mechanisms of PABC. These genes may be used as new molecular targets in the treatment of this disease.

Correspondence to: Dr Hong Yin or Dr Cheng Lu, Department of Breast, Nanjing Maternity and Child Health Care Hospital, Obstetrics and Gynecology Hospital Affiliated to Nanjing Medical University, 123 Mochou Road, Tianfeixiang, Nanjing, Jiangsu 210004, P.R. China
E-mail: yinhhshsh@hotmail.com
E-mail: chengludhh@163.com

*Contributed equally

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Introduction

Pregnancy-associated breast cancer (PABC) is a primary breast cancer characterized by breast lumps, breast thickening and changes in breast shape or pits of skin (1). It occurs during pregnancy or within one year postpartum, with an increased risk of metastasis and mortality. The incidence rate of PABC is ~0.76-3.80% and the age is 23-47 years old (2). The incidence of PABC has risen due to women delaying childbearing (3). Despite efforts being made to improve the diagnosis and treatment of PABC, the development of clinically validated detection markers represents a great challenge. Therefore, it is important to understand the underlying molecular mechanisms of PABC to provide the treatment basis for patients with PABC.

Multiple epidemiological studies have reported that the genetic factors, including breast cancer 1, early onset (*BRCA1*) or *BRCA2* mutations, are involved in increasing the incidence of PABC (4-6). Overexpression of tumor protein p53 and Erb-B2 receptor tyrosine kinase 2 (*ERBB2*) in patients with PABC has been demonstrated to lead to high proliferation of the tumors (7). Furthermore, C-X-C motif chemokine ligand 13 (*CXCL13*) and C-C motif chemokine ligand 20 (*CCL20*) have also been demonstrated to be associated with the progression of PABC (8). Although potential biomarkers associated with PABC have been studied previously, it is far from enough for the diagnosis and treatment of PABC.

The breast consists of two major cell populations, stromal and epithelial cells, that communicate with each other through the extracellular matrix (9). Breast cancer manifests in the epithelium, but the involvement of stromal cells in tumorigenesis is also receiving attention (10). In the present study, gene expression profile data GSE31192 was downloaded to identify the differentially expressed genes (DEGs) between tumor associated stromal cells and normal stromal cells in PABC. Based on the obtained DEGs, Gene Ontology (GO) functional

annotation, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis and protein-protein interaction (PPI) network analysis were performed. Notably, in order to validate the findings of the present study, microarray data of GSE15852 were downloaded, and the DEGs between 43 breast tumors and their paired normal controls were identified. These DEGs were subsequently compared with those in GSE31192. The aim of the present study was to explore the DEGs in tumor-associated stroma of PABC using bioinformatics methods.

Data and methods

Affymetrix microarray data. The unstandardized gene expression profile microarray data GSE31192 was downloaded from the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) (11) database of the National Center for Biotechnology Information, which was deposited by Harvell *et al* (9). The data platform was Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix, Inc.; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The dataset included 33 samples that were obtained from women whose tumors arose while they were pregnant or within 1 year of delivery, and from age-matched controls who had never been pregnant. In the present study, 7 samples of tumor associated stromal cells and 4 samples of normal stromal cells in patients with PABC were selected to study the differences in gene expression.

Data preprocessing and DEGs analysis. All raw data were quantile normalized using the Robust Multiarray Averaging (RMA) method (12) in the Affy version 1.52.0 (http://www.bioconductor.org/packages/release/bioc/html/affy.html) (13) package in R language. In addition, preprocessing including RMA background correction and conversion of probe ID to gene symbol were performed. Eventually, the standardized gene expression matrix of samples was obtained. The DEGs between tumor associated stromal cells and normal stromal cells in PABC patients were analyzed with Limma version 3.30.11 (http://www.bioconductor.org/packages/release/bioc/html/limma.html) (14) package in R language. Student's t-tests were used to obtain the corresponding P-value of the gene symbols. P<0.01 and $\log_2$ fold change (FC) >1were considered as the cut-off values.

GO and KEGG pathway enrichment analyses. GO (http://www.geneontology.org) database (15) is a tool for the functional unification of large-scale genomic data, which includes 3 categories: Biological process (BP), molecular function and cellular component. The KEGG (http://www.genome.ad.jp/kegg/) (16) database is used to classify correlating gene sets into their respective pathways. In the present study, the GO function and KEGG pathway enrichment analyses for the DEGs were performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID version 6.8; https://david.ncifcrf.gov/) (17) online tool, which aimed to provide functional explanations of a large number of genes derived from genomic studies and proteins. With the enrichment thresholds of modified fisher exact P-value <0.05 and the count number >2, the DEG enrichment results in the GO BP and KEGG pathway were obtained (16).

PPI network construction. Search Tool for the Retrieval of Interacting Genes (STRING version 10.0; http://string.embl.de/) (18) is a network resource and biological database of predicted and known PPIs. In this tool, the data of confidence scores were calculated for all protein interactions (19). In the present study, the DEGs were mapped into the STRING database to identify significant protein pairs with a combined score >0.4. Then, the PPI network was constructed based on the obtained PPI interaction pairs. Previous studies on biological networks have revealed that most PPI networks obeyed the scale-free attribution. Thus, the connectivity degree was analyzed using the network statistics to obtain the critical nodes, namely hub proteins (20).

Data validation based on GSE15852. In order to validate the reliability of the results, the expression profile microarray GSE15852 was downloaded from the GEO database based on the platform of GPL96 (HG-U133A) Affymetrix Human Genome U133A (Affymetrix Inc.; Thermo Fisher Scientific, Inc.). There were 86 samples in this dataset, including 43 breast tumors and their paired normal control.

The procedures of data preprocessing were the same as those detailed above. The DEGs between the 43 breast tumors and their paired normal control were then identified with the thresholds of P<0.01 and $\log_2$ FC >1. The identified DEGs were subsequently compared with the DEGs identified in GSE31192.

Results

DEG analysis. In order to obtain DEGs between tumor-associated stromal cells and normal stromal cells in patients with PABC, the publicly available microarray dataset was downloaded from GEO and analyzed with the Limma package. With P<0.01 and $\log_2$ FC >1, a total of 480 genes were identified, including 94 upregulated genes and 386 downregulated ones.

GO enrichment analysis of DEGs. To investigate the functional changes in the pathologic course of PABC, the identified DEGs were mapped to the GO database. Tables I and II list the top 10 GOBP enrichment results of upregulated and downregulated DEGs, respectively. The upregulated DEGs were primarily enriched in BP terms associated with the immune response (12 genes), regulation of cell proliferation (10 genes) and defense response (9 genes). For instance, interleukin 18 (*IL18*) and cluster of differentiation 274 (*CD274)* were primarily enriched in the immune response. The downregulated DEGs were enriched in cell surface receptor linked signal transduction (49 genes), cell adhesion (45 genes) and biological adhesion (45 genes).

KEGG pathway enrichment analysis of DEGs. To gain further insights into the changes of biological pathways in PABC, significant enrichment of the DEGs in was observed in multiple KEGG terms. The significant pathway enrichment results were listed in Tables III and IV. As revealed in Table III, only one significant pathway enriched by upregulated DEGs was obtained: The cytokine-cytokine receptor interaction pathway. In addition, 9 pathways of downregulated DEGs were obtained, including focal adhesion (21 genes), pathways

Table I. GO biological process functional enrichment analysis results of upregulated genes (top 10).

Category	Term	Description	Count	P-value
BP	GO:0006955	Immune response	12	$8.59x10^{-5}$
BP	GO:0042127	Regulation of cell proliferation	10	$4.15x10^{-3}$
BP	GO:0006952	Defense response	9	$3.21x10^{-3}$
BP	GO:0007267	Cell-cell signaling	8	$1.04x10^{-2}$
BP	GO:0042981	Regulation of apoptosis	8	$4.32x10^{-2}$
BP	GO:0043067	Regulation of programmed cell death	8	$4.52x10^{-2}$
BP	GO:0010941	Regulation of cell death	8	$4.60x10^{-2}$
BP	GO:0051249	Regulation of lymphocyte activation	6	$3.28x10^{-4}$
BP	GO:0002694	Regulation of leukocyte activation	6	$5.55x10^{-4}$
BP	GO:0050865	Regulation of cell activation	6	$7.05x10^{-4}$

Category represents GO functional classification. GO, Gene Ontology; BP, biological process.

Table II. Gene Ontology biological process P functional enrichment analysis results of downregulated genes (top 10).

Category	Term	Description	Count	P-value
BP	GO:0007166	Cell surface receptor linked signal transduction	49	$1.14x10^{-2}$
BP	GO:0007155	Cell adhesion	45	$9.72x10^{-13}$
BP	GO:0022610	Biological adhesion	45	$1.01x10^{-12}$
BP	GO:0007610	Behavior	29	$5.78x10^{-8}$
BP	GO:0010033	Response to organic substance	28	$4.60x10^{-4}$
BP	GO:0042127	Regulation of cell proliferation	28	$1.70x10^{-3}$
BP	GO:0042981	Regulation of apoptosis	27	$4.56x10^{-3}$
BP	GO:0043067	Regulation of programmed cell death	27	$5.18x10^{-3}$
BP	GO:0010941	Regulation of cell death	27	$5.41x10^{-3}$
BP	GO:0010604	Positive regulation of macromolecule metabolic process	27	$1.01x10^{-2}$

Category represents GO functional classification. GO, Gene Ontology; BP, biological process.

Table III. KEGG pathway enrichment analysis results of upregulated genes.

Category	Term	Description	Count	P-value
KEGG	hsa04060	Cytokine-cytokine receptor interaction	6	$7.94x10^{-3}$

Category represents KEGG pathway. KEGG, Kyoto Encyclopedia of Genes and Genomes.

in cancer (17 genes), ECM-receptor interaction (13 genes), and vascular smooth muscle contraction (7 genes).

PPI network construction. In order to obtain novel insights into protein function, the PPI network was constructed. Using STRING (combined score >0.4), a total of 286 nodes and 811 protein interaction pairs were obtained (Fig. 1). The nodes with degree ≥10 in the PPI network were listed in Table V, including jun proto-oncogene (*JUN*; degree=61), FBJ murine osteosarcoma viral oncogene homolog (*FOS*; degree=50), epidermal growth factor receptor (*EGFR*; degree=48), V-myc avian myelocytomatosis viral oncogene homolog (*MYC*; degree=32), and α-smooth muscle actin (*ACTA2*; degree=21).

Data validation based on GSE15852. Following data validation of the DEGs based on GSE15852 microarray data, the hub genes obtained in GSE31192, including *JUN*, *FOS*, *EGFR*, *MYC* and *ACTA2* were revealed to have the same expression levels as in GSE15852. In addition, *IL18* (enriched in BP terms associated with the immune response) was also upregulated in GSE15852. The identified DEGs in GSE15852 can be obtained from http://pan.baidu.com/share/link?shareid=9888 37841&uk=3125911049.

Discussion

PABC is a malignant tumor that poses a serious threat to women's health (21). Therefore, exploration of the pathogenesis of PABC is urgent. In the present study, based on the predefined thresholds of the software and online tools used, a

Table IV. KEGG pathway enrichment analysis results of downregulated genes.

Category	Term	Description	Count	P-value
KEGG	hsa04510	Focal adhesion	21	$2.29x10^{-8}$
KEGG	hsa05200	Pathways in cancer	17	$3.45x10^{-3}$
KEGG	hsa04512	ECM-receptor interaction	13	$3.84x10^{-7}$
KEGG	hsa05210	Colorectal cancer	8	$3.14x10^{-3}$
KEGG	hsa05215	Prostate cancer	8	$4.34x10^{-3}$
KEGG	hsa04360	Axon guidance	8	$2.96x10^{-2}$
KEGG	hsa04012	ErbB signaling pathway	7	$1.51x10^{-2}$
KEGG	hsa04270	Vascular smooth muscle contraction	7	$4.50x10^{-2}$
KEGG	hsa05222	Small cell lung cancer	6	$4.47x10^{-2}$

KEGG, Kyoto Encyclopedia of Genes and Genomes.

Table V. Nodes with higher connectivity degree in the PPI network of differentially expressed genes (degree ≥10).

Node	Degree	Node	Degree	Node	Degree	Node	Degree
JUN	61	*LPL*	17	*EPHA2*	14	*MMP3*	12
FOS	50	*NOTCH4*	16	*PENK*	13	*OXTR*	12
EGFR	48	*ABL1*	16	*TNC*	13	*CD36*	12
MYC	32	*ACTG2*	16	*EGR2*	13	*PDE7B*	11
EGR1	27	*NGFR*	16	*BDKRB2*	13	*LAMB2*	11
IGF1	23	*COL4A2*	16	*ITGA7*	13	*NES*	11
PDGFRB	22	*PLCB1*	16	*FOSB*	13	*HSD17B6*	10
ACTA2	21	*ATF3*	15	*TCF7L2*	13	*RASL10A*	10
LPAR1	19	*GNRH1*	15	*DUSP1*	13	*MME*	10
VWF	19	*ITGA5*	14	*LPAR5*	13	*EGR3*	10
COL18A1	18	*NR4A1*	14	*EDNRB*	12		
PTGS2	17	*TAC1*	14	*NR4A2*	12		

Node represents specific gene (protein) name (symbol), Degree represents the gene connectivity degree in the PPI network. PPI, protein-protein interaction.

total of 480 DEGs were identified between tumor stromal cells and normal stromal cells in patients with PABC, including *JUN*, *FOS*, *MYC*, *ACTA2*, *IL18* and *CD274*. These genes were primarily enriched in pathways in cancer, and vascular smooth muscle contraction, and in GO BP terms were associated with cell surface receptor linked signal transduction and the immune response. Furthermore, the DEGs of *JUN*, *FOS*, *MYC* and *ACTA2* were hub genes with higher degrees in the PPI network. Notably, these DEGs, with the exception of *CD274*, were validated by the dataset of GSE15852.

By analyzing the DEGs in the PPI network, *JUN*, *FOS*, *MYC* and *ACTA2* were revealed to be hub genes with higher degrees. *JUN* and *FOS* are proto-oncogenes which are involved in multiple tumors. *JUN* is the component of transcription factor activator protein 1 (AP-1), which primarily forms heterodimers with *FOS*, and was the first oncogenic transcription factor to be discovered (22). AP-1 participates in multiple cellular processes, including cell proliferation, transformation and death, and is also involved in tumorigenesis, proliferation and metastasis. *JUN* is activated by c-jun N-terminal kinases (JNKs) through c-jun N-terminal phosphorylation of the JNK pathway (23). The binding of *JUN* and *FOS* to a high-affinity AP-1 binding site on DNA induces gene transcription and promotes tumorigenesis (24). Previous studies have demonstrated that human chorionic gonadotropin in the breast cancer Michigan Cancer Foundation-7 cell line exerts anti-proliferative and anti-invasive effects by downregulating nuclear factor-κB and AP-1 transcription factors, so as to induce a protective effect in pregnant women (25). Notably, a previous study has also reported that deregulation of *JUN* expression results in the metastasis of breast cancer (26). Therefore, deregulation of *JUN* and *FOS* maybe involved in the tumor-associated stroma of PABC.

MYC is a regulator gene coding for a transcription factor that is involved in cell cycle progression, apoptosis and cellular transformation (27). It is activated by a variety of mitogenic signals, including EGF, wingless and INT-1 via the mitogen-activated protein kinases/extracellular signal-regulated kinases pathway (28). Imbalanced expression of *MYC* promotes cell transformation from G1 to S phase, thereby

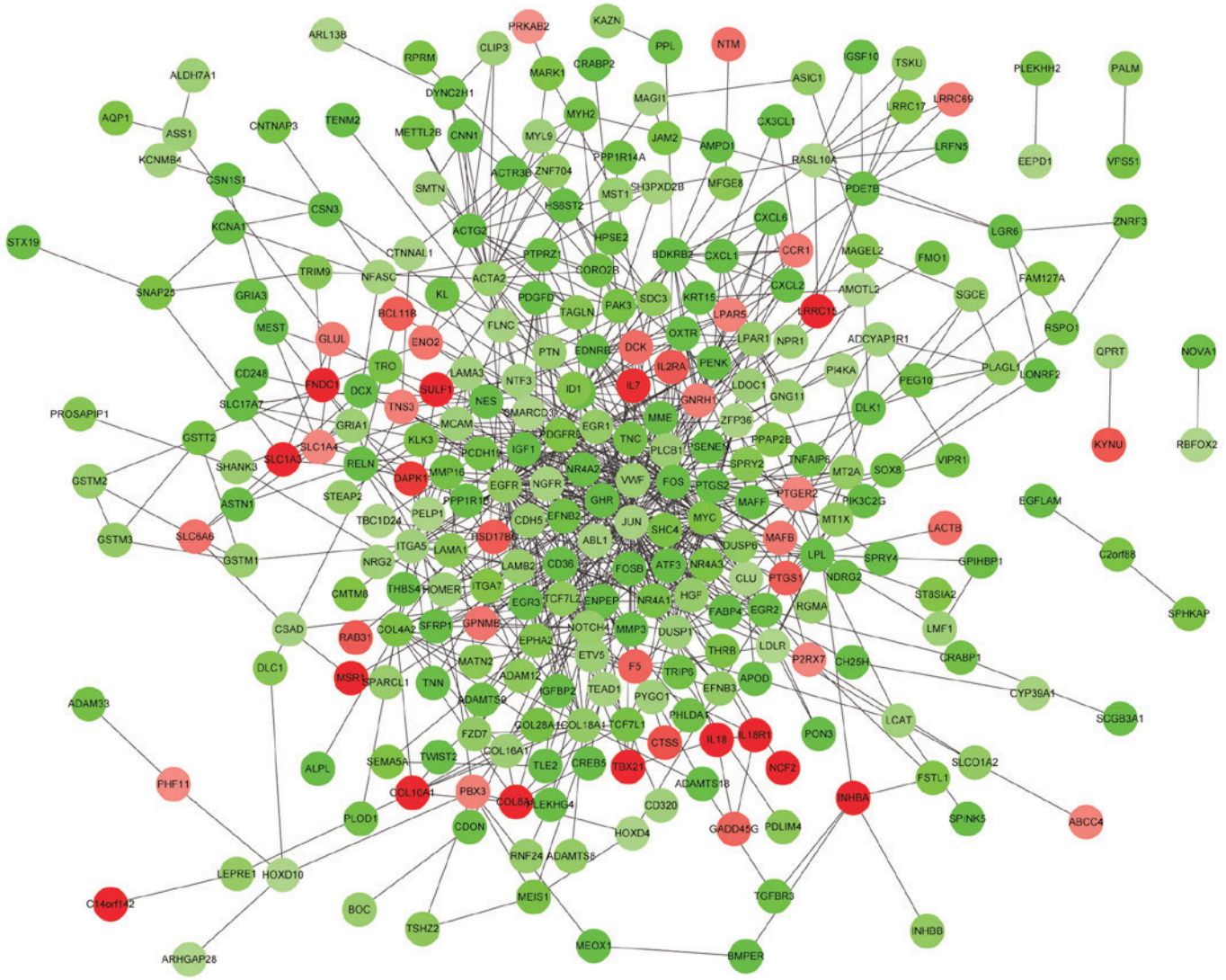

Figure 1. Protein-protein interaction network for the differentially expressed genes in tumor-associated stromal cells of pregnancy-associated breast cancer. There were 286 nodes and 811 edges (protein interaction pairs) in the network. The red nodes represent upregulated genes and the green nodes represent downregulated genes. The color depth represents the strength of significance. The deeper color represents a smaller P-value compared with the normal cells.

leading to cell proliferation and the formation of cancer (29). Notably, malfunctions in *MYC* have been observed in several types of cancer, including breast, uterine, gastric, pancreatic and colorectal cancer (30). *MYC* is thus considered to be a promising target for anti-cancer drugs. Taken together, it was hypothesized that *MYC* may act as a potential biomarker in tumor-associated stroma of PABC.

Another hub gene, *ACTA2*, encodes a protein which belongs to the highly conserved actin family. It is known to contribute to cell-generated mechanical tension and be involved in cell motility, structure and integrity (31). In the present study, *ACTA2* was demonstrated to be enriched in the pathway of vascular smooth muscle contraction. Lambrechts *et al* (32) revealed that *ACTA2* is primarily expressed in the smooth muscle cells and activated cancer-associated fibroblasts. Tumor cells use actin bundles to allow them to break away from a primary tumor and invade the surrounding tissue (33). *ACTA2* has been demonstrated to be involved in lung adenocarcinoma metastasis (31). Therefore, the deregulation of *ACTA2* in tumor stroma may affect the progression of PABC.

Furthermore, previous studies have demonstrated that the expression changes of genes associated with the immune response contribute to tumor aggressiveness (34). The analysis data of Ma *et al* (35) revealed that the upregulated genes associated with the immune response are exhibited in the stroma of high-grade PABC. In the present study, the immune response was a significant BP term that was enriched by *IL18* and *CD274*. *IL18* is a tumor inhibiting factor, dysregulation of which has been observed in the progression of tumors. Studies have reported that *IL18* effectively inhibits the growth of hepatoma cells by inhibiting angiogenesis and participating in apoptotic signal transduction (36). An analysis of tumor specimens with renal cell carcinoma has revealed that high expression of *CD274* increases tumor aggressiveness and risk of death (37). Thus, *IL18* and *CD274* may affect the progression of PABC through the pathways associated with immune response.

However, the present study has limitations. In the process of data analysis, only a few samples were used. In addition, no microarray experiments were performed, except for bioinformatics analysis of two gene expression profile data from the

GEO database. Therefore, further experimental studies with larger sample size are required to confirm the observations of the present study.

In conclusion, there were significant differences in gene expression between tumor-associated stromal cells and normal stromal cells in PABC, confirmed by analyzing the gene expression profiles with bioinformatics. The DEGs of *JUN*, *FOS*, *MYC*, *ACTA2*, *IL18* and *CD274*, and BP terms associated with the immune response may be involved in the development of tumor stroma in PABC.

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