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Expression profile analysis for epithelial-mesenchymal transition of breast cancer cell line DKTA based on microarray data

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Summary

Objective: This study aimed to explore the molecular mechanisms of epithelial-mesenchymal transition (EMT) in breast cancer cells. *Materials and Methods:* GSE33146 microarray data downloaded from Gene Expression Omnibus (GEO). Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of differentially expressed genes (DEGs) was performed, followed by functional annotation. Protein-protein interaction (PPI) network was constructed, and then five modules were mined for functional analysis. *Results:* A total of 507 DEGs including 229 up- and 278 down-regulated DEGs were screened between pre- and post-EMT samples. The screened DEGs mainly enriched in KEGG pathways, such as focal adhesion (p = 0.017), TGF-beta signaling pathway (p = 0.028), and ECM-receptor interaction (p = 6.89E-04), and also enriched in some GO terms such as regulation of cell proliferation (p = 2.48E-06) and ectoderm development (p = 2.76E-08). By functional analysis of DEGs, a total of ten proto-oncogenes including *PBX1, MYBL1, MET, VAV3,* and *MYC*, and 42 anti-oncogenes including *TXNIP, TPM1, TMEFF2, TP63,* and *STEAP3* were obtained. Seven identified DEGs including *SNCG, PTHLH, OAS1*, *KRT5, ITGB4, CHRM3,* and *CBLB* were obtained. The constructed PPI network contained 192 nodes and 293 edges. A total of five models were screened from PPI network. DEGs in five modules were enriched in various functions, such as response to virus (FDR = 15.2), G-protein coupled receptor protein signaling pathway (FDR = 16.21), focal adhesion (FDR = 5.52E-05), and ECM-receptor interaction (FDR = 6.56E-07). *Conclusions:* The identified DEGs, especially in five modules, such as *OAS1, IFI27, LPAR1, PTGFR, ITGB4,* and *ITGA6* might participate in EMT process for breast cancer cell lines DKTA.

Key words: Breast cancer; Epithelial-mesenchymal transition; PPI network.

Introduction

Breast cancer, the most common malignancy in women, is a collection of diseases with distinct genetic and genomic variability, histopathological features, and diverse prognostic outcomes [1, 2]. Breast cancer cells can invade by migration and retain their epithelial characteristics including apical-basal polarity and adherens junctions [3, 4]. In addition, epithelial-mesenchymal transition (EMT) also plays an important role in tumor metastasis and invasion [5].

EMT is a biological process beginning by the loss of E-cadherin, with the steps including epithelial cells lose their cell-cell adhesion, gain migratory and invasive properties, and finally become mesenchymal stem cells [6, 7]. Many transcription factors such as *ZEB1, E47, SIX1,* and *FOXC2* can regulate the expression of E-cadherin directly or indirectly [8]. These TFs not only regulate the expression of E-cadherin, but also repress other junctional proteins thus facilitating EMT [9]. Furthermore, several signaling pathways including Ras-MAPK, TGF-β, EGF, HGF, and Notch may induce EMT [10]. Among of these, Ras-MAPK was confirmed to activate Slug which triggers the processes including cell spreading, desmosomal disruption, and partial separation at cell-cell borders [11]. At the same time, Snail is also known to be activated by Ras-MAPK. Both Snail and Slug regulate the expression of p63, which plays a key role in reducing cell-cell adhesion and increasing the migratory properties of cancer cells [12]. In breast cancer cells, activation of Wnt pathway upregulates the mesenchymal marker vimentin and reduces the EMT regulator SNAIL [13]. In addition, the Wnt pathway is closely related with poor prognosis in breast cancer patients. Smith *et al.* showed the correlation between Six1 and miR-106b, and further activated TGF-β in human breast cancers, by which TGF-β signaling shifts from tumor suppressive to tumor promoting [14]. Although EMT has been researched for many years, the signal transduction pathway and molecular mechanism related to EMT in breast cancer are yet not definitive.

The present study aimed to reveal the underlying molecular mechanism of EMT in breast cancer cells. In this present study, expression microarray data of breast cancer cell lines DKAT cultured in different medium conditions was used to screen differently expressed genes (DEGs) for func-

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tional and pathway analysis. In addition, gene functional annotation was applied for extracting known proto-oncogene and anti-oncogenes. Furthermore, protein-protein interaction (PPI) network was constructed, and then sub-network was mined from PPI network.

Materials and Methods

All studies have been approved by the Chinese PLA General Hospital Ethics Committee. The gene expression profile of GSE33146 was obtained from Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/). The platform was Affymetrix Human Genome U133 Plus 2.0 Array. A total of six DKAT samples were available under different culture conditions. GSM820817, GSM820818, and GSM820819 were the repeated DKAT samples under culture conditions with mammalian epithelial cell medium (serum-free, morphology of epithelial cells). Meanwhile, GSM820820, GSM820821, and GSM820822 were the repeated DKAT samples under culture conditions with cell substrate cell medium (serum, morphology of interstitial cells)

The raw data were read by Affy [15] package in bioconductor and processed by Affy annotation files of chip probe in Brain Array Lab. The preprocessing steps included RMA [16] background correction, quantile normalization, and probe summarization, and then the gene expression matrix of samples was obtained. In addition, *t*-test was applied for calculation and analysis of DEGs between pre- and post-EMT group. Genes with FDR < 0.05 and $\log FC| > 1$ were selected as DEGs. At the same time, the FC coefficient ≥ 2 between different groups was the threshold.

Database for Annotation, Visualization and Integrated Discovery (DAVID) is a web based tool for extracting biological meaning from large list of genes [17]. It was used for Gene ontology (GO) function [18] and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways [19] enrichment analysis of up- and downregulated DEGs. $P < 0.05$ was chosen as the cut off criterion.

Combined with the information of transcription factors, DEGs were screened and annotated to ensure the transcriptional regulatory function. All known proto-oncogene and anti-oncogenes was further screened by combining the cancer genes databases including TS gene [20] and TAG [21].

Search Tool for Retrieval of Interacting Genes (STRING) database was an database to provide integrated knowledge of the known and predict associations for protein network [22]. Based on this network, protein-coding genes which could interact with specific genes were collected and PPI network was constructed. The modules were constructed by pairwise protein information which derived from experimental verification, data mining, co-expression analysis, and database records. Moreover, two genes inserted in pairwise protein were DEGs with combine score > 0.7. Subnetwork was mined from PPI network of DEGs based on CFinder [23] profiler.

Results

Based on the differential expression analysis for GSE33146, the authors obtained a total of 1,656 differential expressed transcripts with FDR < 0.05 and $\log FC| > 1$. Among them, 799 upregulated transcripts were corresponding to 229 DEGs, while 857 downregulated transcripts corresponded to 278 DEGs. Figure 1 is the scatterplot and volcano plot for expression profile data of DKAT in pre- and post-EMT groups.

Table 1. — *The results of KEGG pathway enrichment analysis for up- and downregulated DEGs after EMT.*

A up-regulated DEGs		
Term	Count	*p* value
Focal adhesion	8	0.017083
TGF-beta signaling pathway	5	0.028396
B down-regulated DEGs		
Term	Count	*p* value
ECM-receptor interaction	8	6.89E-04
Small cell lung cancer	7	0.003693
Pathways in cancer	14	0.005172
Focal adhesion	9	0.026582
p53 signaling pathway	5	0.032778
Steroid biosynthesis	3	0.036188
Aldosterone-regulated sodium reabsorption	4	0.036235

Table 2. — *Functional enrichment analysis for up- and downregulated DEGs after EMT (first ten terms).*

A: Upregulated DEGs.		
Term	Count	*p* value
GO:0042127~regulation of cell proliferation	27	2.48E-06
GO:0008285~negative regulation of cell proliferation	16	3.09E-05
GO:0060429~epithelium development	12	9.56E-05
GO:0045597~positive regulation of cell differentiation	12	1.03E-04
GO:0043542~endothelial cell migration	5	1.25E-04
GO:0060284~regulation of cell development	11	1.89E-04
GO:0006928~cell motion	17	2.00E-04
GO:0030155~regulation of cell adhesion	9	2.51E-04
GO:0048729~tissue morphogenesis	10	3.31E-04
GO:0040012~regulation of locomotion	10	5.30E-04
B: Downregulated DEGs.		
Term	Count	*p* value
GO:0007398~ectoderm development	18	2.76E-08
GO:0008544~epidermis development	16	3.42E-07
GO:0007155~cell adhesion	28	2.90E-05
GO:0022610~biological adhesion	28	2.97E-05
GO:0042127~regulation of cell proliferation	28	2.07E-04
GO:0060429~epithelium development	13	3.58E-04
GO:0030855~epithelial cell differentiation	10	4.14E-04
GO:0045616~regulation of keratinocyte differentiation	4	6.44E-04
GO:0030856~regulation of epithelial cell differentiation	5	0.001194591
GO:0045604~regulation of epidermal cell differentiation	4	0.001370493

By KEGG pathway enrichment analysis, a total of two pathways, upregulated DEGs enriched in two pathways (Table 1A), including focal adhesion ($p = 0.017$) and TGF-beta signaling pathway ($p = 0.028$). At the same time, downregulated DEGs enriched in seven pathways, such as ECM-receptor interaction ($p = 6.89E\text{-}0.4$), small cell lung cancer ($p = 0.003693$), pathways in cancer ($p = 0.005172$), focal adhesion ($p = 0.026582$), and p53 signaling pathway ($p = 0.032778$) (Table 1B). The most significant pathway

Table 3. — *Transcription factor and cancer related genes in DEGs after EMT.*

	TF	Counts
Up	FOXA1, FOXD1, HOXA1, HOXA4, HOXA5, ID1, ID2, ID3, MEIS2, MITF, MYBL1, NR1D2, NR2F2, NR3C2, PBX1, PPARG, SMAD3, TGFB1I1	18
Down	AHR, ARNTL, EGR2, EGR3, FOSB, FOXI1, GLI3, IRX4, IRX5, KLF7, NFIA, TP63	12
	TAG	Counts
Up	PBX1, MYBL1, MET, LCN2, FGF2, CCND1	6
Down	TXNIP, TPM1, TMEFF2, TGFBI, SRPX, SMAD3, SLIT2, RARRES3, RARRES1, PPP1R3C, PLK2, PLA2G16, MTUS1, LXN, LOX, IGFBP3, GPX3, GPRC5A, FAT4, DLC1, DAB2, CST6, CDKN2C, CDH13	24

was ECM-receptor interaction, which enriched by downregulated DEGs including *LAMB3, SDC1, LAMA3, ITGA6, ITGB6, ITGB4, LAMC2,* and *COL4A6*. Furthermore, some GO terms, such as regulation of cell proliferation (p = 2.48E-06), negative regulation of cell proliferation (p = 3.09E-05), and epithelium development (p = 9.56E-03), were enriched by upregulated DEGs (Table 2A). Downregulated DEGs also enriched in some GO terms including ectoderm development (p = 2.76E-08), epidermis development (p = 3.42E-07), and cell adhesion (p = 2.90E-05) (Table 2B). The most significant GO term was ectoderm development, which enriched by downregulated DEGs such as *KRT6A, KRT6B, GJB3, TP63, GJB5, SFN, JAG1, PTHLH,* and *LAMB3*.

EMT process effected breast cancer cell line DKAT by effecting the expression of various transcription factors and TAGs. After EMT process, the authors obtained 18 significantly upregulated TFs including *FOXA1, FOXD1, HOXA1, HOXA4, HOXA5, ID1,* and *ID2*. At the same time, a total of 12 significantly downregulated TFs, such as *AHR, ARNTL, EGR2, EGR3, FOSB, FOXI1,* and *GLI3*, were screened (Table 3A). In addition, Table 3B shows the known TAGs which were screened from DEGs. In upregulated DEGs, a total of six proto-oncogene including *PBX1, MYBL1, MET, LCN2, FGF2,* and *CCND1* were detected, while a total of 24 anti-oncogenes, such as *TXNIP, TPM1, TMEFF2, TGFBI, SRPX, SMAD3, SLIT2,* and *RARRES3*, were found. Similarly, in downregulated DEGs, a total of four proto-oncogene including *VAV3, MYC, LAMC2,* and *FOS* were obtained, while 18 anti-oncogenes, such as *TP63, STEAP3, SIK1, SFN, SERPINB5,* and *S100A2*, were detected. The authors obtained seven known DEGs including *SNCG, PTHLH, OAS1, KRT5, ITGB4, CHRM3,* and *CBLB*, which were tumor-associated, but with an unclear mechanism.

The PPI network, which contained 192 nodes and 193 edges, was constructed. Based on the degree of the nodes, top ten upregulated hub nodes with higher degrees were

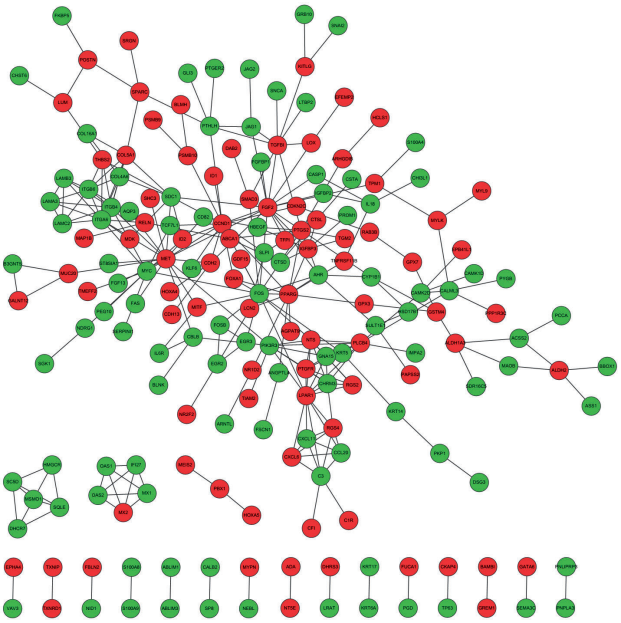

Figure 1. — PPI network of DEGs. Red and green nodes represent up- and downregulated DEGs, respectively.

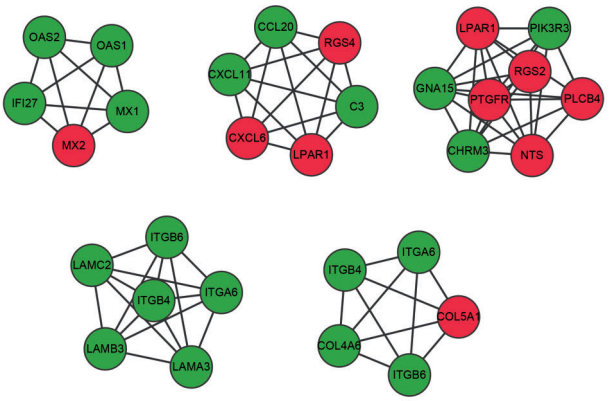

Figure 2. — Functional modules of breast cancer DKAT after EMT screened from PPI network. Red and green nodes represent up- and downregulated DEGs, respectively.

screened, including *FOS, FGF2, MET, CCND1, LPAR1, PPARG, ITGA6, ITGB4, MYC,* and *PTGS2*. Among of them, *FGF2* showed the highest node degree of 16 (Figure 1).

A total of five subnet modules, which K_CKUQUE parameter was 5, were obtained (Figure 2). Module 1 contained one upregulated DEG (*MX2*) and four downregulated DEGs (*IFI27, OAS2, OAS1,* and *MX1*). This module participated in the GO term of response to virus infection. The result of GO analysis was in accordance with the enrichment analysis of protein domain family. Moreover, module 2 was constituted by three upregulated DEGs (*RGS4, CXCL5* and *LPAR1*) and three downregulated

DEGs (*C3, CXCL11* and *CCL20*). The DEGs in module 2 were enriched in several GO terms (inflammatory response, G-protein coupled receptor protein signaling pathway, and response to wounding) and KEGG pathways (chemokine signaling pathway and cytokine-cytokine receptor interaction). Five upregulated DEGs (*LPAR1, RGS2, PTGFR, PLCB4,* and *NTS*) combined with three downregulated DEGs (*GNA15, CHRM3,* and *PIK3R3*) constituted module 3. The DEGs in module 3 were enriched in some important GO terms (muscarinic acetylcholine receptor signaling pathway, cell surface receptor linked signal transduction, and G-protein coupled receptor protein signaling pathway) and KEGG pathways (calcium signaling pathway and neuroactive ligand-receptor interaction), while the protein domain family enriched in terms including GPCR, rhodopsin-like superfamily, 7TM GPCR, and rhodopsin-like. In addition, module 4 contained five downregulated DEGs (*LAMC2, ITGB6, ITGA6, LAMA3, LAMB3,* and *ITGB4*), while module 5 contained one upregulated DEG (*COL5A1*) and five downregulated DEGs (*ITGB4, ITGA6, COL4A6,* and *ITGB6*). DEGs in modules 4 and 5 were mainly enriched in GO terms including cell adhesion, biological adhesion, and integrin-mediated signaling pathway. At the same time, they also enriched in KEGG pathways including ECM-receptor, interaction, and focal adhesion.

Discussion

Breast cancer is a common cancer among women, however it still remains difficult to be overcome [24]. In this study, GSE33146 was downloaded from GEO to research the molecular mechanism of EMT in human breast cancer. Total 507 DEGs including 229 up- and 278 downregulatory DEGs were screened in the EMT process. The DEGs significant enriched into the pathways, such as focal adhesion, TGF-beta signaling pathway, and ECM-receptor interaction. At the same time, they also enriched in GO terms including regulation of cell proliferation, negative regulation of cell proliferation, and ectoderm development. Based on the analysis of DEGs, a total of ten proto-oncogenes, 42 anti-oncogenes, and seven undefined genes were obtained. PPI network with 192 nodes and 293 edges was constructed in this study. Based on module partition of PPI network, five modules involving 30 DEGs were obtained for GO and KEGG pathway analysis.

In the present study, the authors found six genes (*IFI27:* interferon, alpha-inducible protein 27*, OSA1:* AT rich interactive domain 1B*, LPAR1:* lysophosphatidic acid receptor 1, *PTGFR:* prostaglandin F receptor*, ITGB4:* integrin, beta 4 and *ITGA6:* integrin, alpha 6) that were previous reported to be differently expressed in EMT process. *IFI27*, an encoded interferon alpha-inducible protein, was confirmed to be unregulated in various cancers, including ovarian carcinoma, hepatocellular carcinoma, and breast cancer [25]. *OSA1*, also known as *ARID1B*, is a BRG1-binding protein-coding [26]. A previous study showed that interferon-inducible including *OSA1* and *IFI27*, combined with the expression of transcription factors *STAT1* and *STAT2*, activate interferon a/brelated signaling pathways [27]. Furthermore, *OSA1* was an important component of SWI/SNF complex which mediates downstream *TGFβ* signaling [28]. Tumor activity could be regulated by TGFβ signaling which is the capacity to induce EMT [29]. In this study, both *OSA1* and *IFI27* were screened in module 1, and might be participated into the EMT process of breast cancer by enriched in the function of respond to virus. Furthermore, *LPAR1* was screened with the degree of 13 and participated into EMT by inflammatory response and chemokine signaling pathway in module 2. *LPAR1* encode lysophosphatidic acid receptor 1, which binds the lipid signaling molecule lysophosphatidic acid [30]. In addition, the internalization of N-cadherin downstream of *LPAR2* could regulate the level of cell-cell adhesion, results affecting the EMT process [31]. Jahn *et al.* [32] found that post-EMT cancer cells upregulated LAPA receptors and acquired increased responsiveness to LAPA. Thereby, *LPAR1* might play a key role in EMT process in breast cancer. Besides, *PTGFR*, a member of G-protein coupled receptor family, is known to play a key role in EMT of breast cancer, but the function related to cancer is not clear [33]. In this study, *FTGFR*, associated with other DEGs in modules 3, enriched in GRCR signaling pathway to induce the EMT process. Therefore, *FTGFR* might a key gene in EMT of breast cancer. Parallel results, *ITGB4* and *ITGA6* existed in module 4 and 5 concurrently. They all enriched in focal adhesion and ECM-receptor interaction. *ITGB4*, encoded the integrin beta 4 subunit, a receptor for laminin [34]. Ferraro *et al.* showed that *ITGB4* is remarkably downregulated after miR-21 overexpression and depressed after transient miR-21 silencing, further regulating the process of EMT [35]. In addition, the stable one known as ZEB1 resulted in an increase in transwell migration, and ZEB1 directly interacts with promoter of *ITGB4* [36]. Similarly, *ITGA6*, encoded integrin alpha-6 protein, always participates in cell adhesion and cell-surface mediated signaling together with beta 4 and beta 1 [37]. Through observation of proteins related to EMT, *ITGA6* was found to be involved in the regulation of focal adhesion [38]. Moreover, in the process of EMT induced by *TGFβ*, focal adhesion kinase signaling is required for invasiveness and mesenchymal markers and E-cadherin delocalization of membrane-bound [39]. Thereby, *ITGB4* and *ITGA6* might be the potential key genes in the EMT process of breast cancer.

Conclusion

In conclusion, the identified DEGs, especially in five modules, such as *OSA1, IFI27, LPAR1, PTGFR, ITGB4,* and *ITGA6* might participate in EMT process for breast

cancer cell lines DKTA. However, these results need to be further confirmed by experimental study.

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