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Analysis of differentially expressed genes in ductal carcinoma with DNA microarray

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Abstract. – AIM: The aim of this study is to investigate the dysregulated biological functions that play important role in the occurrence and development of breast invasive ductal carcinoma (IDC).

MATERIALS AND METHODS: We downloaded the gene expression profile data from gene expression omnibus (GEO) database, including 42 disease samples and 143 adjacent histological normal samples. Significance analysis of microarrays (SAM) was employed to identify differentially expressed genes (DEGs) between the normal and disease samples. Gene ontology (GO) function enrichment analysis was based on Software DAVID, followed by KEGG pathway enrichment analysis. TRANSFAC database and HPRD database were employed to construct the transcriptional regulatory network (Tnet) and protein-protein interaction (PPI) network, respectively.

RESULTS: We got a total of 1769 genes significantly differentially expressed, including 907 up-regulated genes and 862 down-regulated genes. Functional analysis revealed that hormone-responsive genes are related with the occurrence of cancer. Then, we successfully constructed IDC-specific Tnet and PPI network with DEGs response to hormone and obtained some hub genes, such as FOS and PIK3R1, in these networks. Besides, ten modules were found in these networks.

CONCLUSIONS: Hormone-responsive genes and modules may play an important role in the occurrence and development of IDC. Based on the findings above, we got a preliminary understand of the occurrence, development and metastasis of the IDC and possibly provided effective information on the biogenesis of IDC.

Key Words:

Invasive ductal carcinoma, Differentially expressed genes, Transcriptional regulatory network, Protein interaction network.

Abbreviations

IDC = invasive ductal carcinoma
GO = gene ontology
PPAR = peroxisome proliferator activated receptors
Jak-STAT = Janus Kinase-signal transducers and activators of transcription
MAPK = mitogen-activated protein kinase
ECM = extracellular matrix
DEGs = differentially expressed genes
KEGG = Kyoto Encyclopedia of Genes and Genomes
NCBI = National Center for Biotechnology and Information
PI3K = phosphatidilinositol 3-kinase
SRE = serum response element
SRF = serum response factors

Introduction

Breast cancer is one of the most common malignant cancers among women world-wide. In recent years, the incidence of breast cancer shows an increasing trend in both developed and developing countries[1,2]. With the improvement of diagnostic methods, the mortality of breast cancer has declined, but it still remains the most common cause of cancer deaths and accounts for approximately 15% of cancer-related deaths in women[3-5].

IDC, also known as Infiltrating Ductal Carcinoma/Infiltrating Carcinoma/Invasive breast cancer, is the most common breast cancer diagnosed in women, which accounts for 80 to 85% of all breast cancer diagnosed. IDC suggested that the cancer has broken through the ducts and has invaded neighboring fatty tissues. It was reported that most of IDC, develops through sequential stages, from premalignant hyperplastic breast lesions with or without atypia to breast ductal cancer in situ (DCIS) and then to IDC[6-9]. And there may be an interim stage, named as DCIS with microinvasive (DCIS-Mi), in the progression from primary breast cancer DCIS to metastasis IDC[10,11].

According to the multistep model of carcinogenesis, tumors may develop and progress as a result of accumulation of mutations in oncogenes and tumor suppressor genes[12,13]. Several hormone-related factors, such as age at menarche, parity, and age at menopause, are major risk fac-

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tors in the occurence of breast cancer[14]. Early epidemiological data has indicated that high levels of sex hormones, such as estrogens, are the common reason for breast cancer in postmenopausal women[14-16]. Similarly, sex hormones also play important roles in the pathogenesis and development of IDC of human breast. Recently, different hormone-responsive gene expression profiles are shown to be associated with the progression from primary breast cancer to metastasis. However, there is lack of systematic analysis about the role for hormone-responsive genes in the progression of IDC. And so far, the actual causative mechanisms of IDC development have remained elusive.

In this study, we analyze the DEGs by comparing IDC specimens with normal control tissues through systems biology methods. GO function and KEGG pathway enrichment analysis indicate that most of hormone-responsive genes are cancer related genes. Further, transcriptional regulatory network (Tnet) provides us a collection of hub genes. These hub genes may play essential role in IDC development. And by searching in protein-protein interaction (PPI) network, we got several functional modules which were confirmed by early studies. In conclusion, through systematic analysis, we obtained more information about the role of hormone-responsive genes in the development of IDC.

Materials and Methods

Microarray Data

Publicly available microarray dataset (ID: GSE10780) were obtained from Gene Expression Omnibus (GEO) database which were deposited by Chen et al[17]. Microarray analysis was performed using the Affymetrix U133 Plus 2.0 GeneChips (54,675 probe sets). A total of 185 genechips were available, including 42 genechips of IDC and 143 genechips of normal breast tissues.

The original data were performed background correction and quartile data normalization using Robust Multi-array Average (RMA)[18,19] algorithm. All of the expression values were then converted to fold changes (FC) with $\log_2$ base. Probe sets were mapped to NCBI entrez genes. If there are multiple probe sets that correspond to the same gene, the expression values of those probe sets are averaged. As a result, we got a total of 19803 genes from this dataset.

Screening of DEGs

Significance analysis of microarrays (SAM) was employed to identify statistically significant genes by comparing the expression value of each single gene between the IDC group and the normal control group. To circumvent the multi-test problem which might induce too much false positive results, the Benjamini-Hochberg (BH) method[20] was used to adjust the raw *p*-values into false discovery rate (FDR). Genes with FDR less than 0.001 and fold-change more than 1.5 were selected as DEGs. The DEGs we got were used for further analysis.

GO and KEGG Enrichment Analysis

The Database for annotation, visualization and integrated discovery (DAVID)[21] was used to analyze the biological process terms or pathway of the DEGs in the context of the Gene Ontology (GO) or KEGG with the threshold of EASE score set at 0.1. DAVID is online free software with multifunction tools, and its basic principle is based on the Fisher's exact test.

Tnet Assembling in TRANSFAC

The Transcription factors database (TRANSFAC) (http://www.gene-regulation.com/pub/databases.html#transfac/) is a unique knowledge-base containing published data on eukaryotic transcription factors[22]. Regulatory relationships between TFs and target genes play key roles in genetic regulatory networks. Using the regulation data that have been collected from TRANSFAC database, we matched the relationships between TFs and its target DEGs.

PPI Network Establishing in HPRD

We used human protein reference database (HPRD) (http://www.hprd.org)[23] to construct IDC-specific PPI network and observed the distribution characteristics of DEGs in the network. Then we searched the tight coupling modules employing Clique Percolation Method[24] provided by CFinder software[25]. Modules are biological individuals that can be delineated from their surroundings or context, and whose behavior or function reflects the integration of their parts, not simply the arithmetical sum.

Results

DEGs in IDC Patients

We download publicly available microarray dataset GSE10780 from GEO database and em-

ployed SAM method to identify the genes specifically differentially expressed in IDC specimens versus normal control tissues with the false discovery rate (FDR) < 0.001 and fold change > 1.5 as cut-off criteria. Based on the criteria, a total of 1769 genes were considered to be significantly differentially expressed, including 907 up-regulated genes and 862 down-regulated genes.

GO Analysis

The GO-function algorithm was used for biological process enrichment analysis of the identified 1769 DEGs with the threshold of EASE score < 0.1. Here, the GO-functional annotations of top 20 categories of significantly DEGs are showed in Table I. The altered categories can be grouped into three general types: response to hormone stimulus, cell cycle related, and vascular development related.

To further explore expression profile of hormone-responsive genes, we chose and analyzed the following five categories: (1) response to hormone stimulus, (2) response to endogenous stimulus, (3) response to steroid hormone stimulus, (4) response to estrogen stimulus, and (5) response to peptide hormone stimulus. Total 785 hormone-responsive genes were found in these five categories. And 774 genes are detected on Affymetrix microarray platform, of which 142 genes are differentially expressed, including 57 up-regulated genes and 85 down-regulated genes.

KEGG Pathway Analysis

To further investigate the function of hormone-responsive DEGs, we carried out KEGG pathway enrichment analysis on DEGs with the threshold of EASE score set at 0.1. Eighteen KEGG pathways are found significant changed with $p < 0.1$ (Table II). Most of these pathways are related with cancer, such as Pathways in cancer, Melanoma, Bladder cancer, and Prostate cancer. Many signaling pathways or protein-protein interactions are also discovered, which has been reported to relate with progression of tumors, including PPAR signaling pathway, Chemokine signaling pathway, Jak-STAT signaling pathway, MAPK signaling pathway, Cytokine-cytokine receptor interaction, Focal adhesion, ECM-receptor interaction and Adherens junction.

Tnet Analysis

To clarify the distribution characteristics of these hormone-responsive DEGs in Tnet, we built the network, and from which we try to find the transcription factors and target genes that related with IDC. Among all 142 genes, there are 47 genes in the Tnet, including 34 transcription factors and 13 target genes. Simultaneously, it forms 204 regulatory interactions interrelated with these 47 genes. Finally, we assemble the Tnet with these hormone-responsive genes (Figure 1). The network consists of 47 genes and 204 regulatory interactions. The average degree of the

Table I. GO functional enrichment analysis of 1769 DEGs (Top 20).

Term	Term name	Bonferroni
GO:0010033	Response to organic substance	1.55E-07
GO:0009725	Response to hormone stimulus	3.87E-07
GO:0009719	Response to endogenous stimulus	5.54E-07
GO:0007067	Mitosis	1.03E-06
GO:0000280	Nuclear division	1.03E-06
GO:0048285	Organelle fission	1.62E-06
GO:0000087	M phase of mitotic cell cycle	2.07E-06
GO:0000278	Mitotic cell cycle	8.74E-06
GO:0051301	Cell division	3.00E-05
GO:0022402	Cell cycle process	4.67E-05
GO:0007049	Cell cycle	1.23E-04
GO:0048545	Response to steroid hormone stimulus	1.69E-04
GO:0000279	M phase	3.52E-04
GO:0007059	Chromosome segregation	3.64E-04
GO:0007010	Cytoskeleton organization	4.78E-04
GO:0006928	Cell motion	5.12E-04
GO:0022403	Cell cycle phase	5.21E-04
GO:0043627	Response to estrogen stimulus	5.28E-04
GO:0001944	Vasculature development	9.10E-04
GO:0001568	Blood vessel development	0.001037

Table II. Annotation of 142 DEGs in KEGG pathway.

Name	Annotation genes	*p* value
Insulin signaling pathway	"SOCS2, FOXO1, PCK1, PRKAR2B, IRS1, SORBS1, EIF4EBP1, IRS2, FBP1, PIK3R1"	5.73E-04
Adipocytokine signaling pathway	"LEP, "PCK1, IRS1, ADIPOQ, IRS2, ADIPOR1, CD36"	0.001091
Type 2 diabetes mellitus	"SOCS2, IRS1, ADIPOQ, IRS2, PIK3R1, PRKCD"	0.001326
Pathways in cancer	"ERBB2, PDGFA, MMP9, FOXO1, PTGS2, ACVR1C, PPARG, FGFR3, ARNT2, FGF1, FGF2, FOS, PIK3R1, CDH1"	0.001669
Cytokine-cytokine receptor interaction	"CXCL13, CXCL12, PDGFA, CX3CR1, CCL21, CCR7, LEP, TNFSF4, GHR, NGFR, INHBB, INHBA"	0.006102
PPAR signaling pathway	"SLC27A1, PCK1, ADIPOQ, SORBS1, PPARG, CD36"	0.007166
Melanoma	"PDGFD, PDGFA, FGF1, FGF2, PIK3R1, CDH1"	0.008076
Focal adhesion	PDGFD, COL5A2, ERBB2, PDGFA, SPP1, COL1A, CCND2, COL1A2, CAV1, PIK3R1"	0.008687
ECM-receptor interaction	COL5A2, SPP1, COL1A1, SDC1, COL1A2, CD36"	0.016016
Chemokine signaling pathway	"GNG11, CXCL13, CXCL12, CX3CR1, CCL21, CCR7, GNG5, PIK3R1, PRKCD"	0.016997
Jak-STAT signaling pathway	"SOCS2, SPRY2, SPRY1, LEP, CCND2, GHR, PIK3R1"	0.019253
Aldosterone-regulated sodium reabsorption	"NR3C2, IRS1, IRS2, PIK3R1"	0.035234
Bladder cancer	"ERBB2, MMP9, FGFR3, CDH1"	0.037471
Adherens junction	"ERBB2, SORBS1, ACVR1C, SNAI2, CDH1"	0.046705
Prostate cancer	"PDGFD, ERBB2, PDGFA, FOXO1, PIK3R1"	0.072009
Arachidonic acid metabolism	"PTGDS, PLA2G4A, PTGS2, GPX3"	0.075996
Vibrio cholerae infection	"ATP6V1A, TCIRG1, ATP6V0B, ATP6V1C1"	0.075996
MAPK signaling pathway	"DUSP1, PDGFA, FGF1, PLA2G4A, FGF2, ACVR1C, FOS, DUSP6, FGFR3"	0.098723

network is 4.42. Figure 1 showed the IDC-specific Tnet with the nodes that the degree larger than 3. Furthermore, we sorted out the hub genes which were considered to play important role in development of IDC. According to the nodes degree, we arranged the hub genes with the degree larger than 4.2 in Table III.

PPI Network

In order to seek the abnormal disturbance of functional module in the development of IDC, we assemble PPI network of the hormone-responsive genes using data from HPRD database. We removed self-interaction proteins and mapped corresponding genes to Entrez Gene. The results come to a network including 9518 genes and 37041 PPI. Next, we intersect these genes in Affy microarray platform and it comes out a network containing 37017 PPI. And then, we annotate these 142 differential expressed hormone-responsive genes to the network. We got 119 annotated nodes and 1934 PPI, which form the final protein interaction network with average degree 16.6. The nodes with the degree > 16.6 in the network are showed in Table IV. Furthermore, we attempt to search tight coupling modules in the network using Clique Percolation Method (CPM) provided by CFinder software. Ten modules were found and the structures are presented as Figure 2.

Discussion

In current study, we focus on the hormone-responsive significant DEGs in invasive ductal breast tumors as compared to normal tissues. The signaling system of sex hormones, e.g. estrogen, has long been implicated in the induction and/or promotion of carcinogenesis especially in breast cancer through regulation of the expression of various important cellular oncogenes and tumor suppressor genes. We try to find crucial genes and biological function modules strongly associated with IDC from system biology standpoint.

Studies using global gene expression profiling have revealed that sex hormones regulate the expression of genes involved in various cellular functions, including signal transduction, cellular proliferation, apoptosis and motility[26-28], which are highly related with the development of cancer. In our GO clustering analysis for the DEGs,

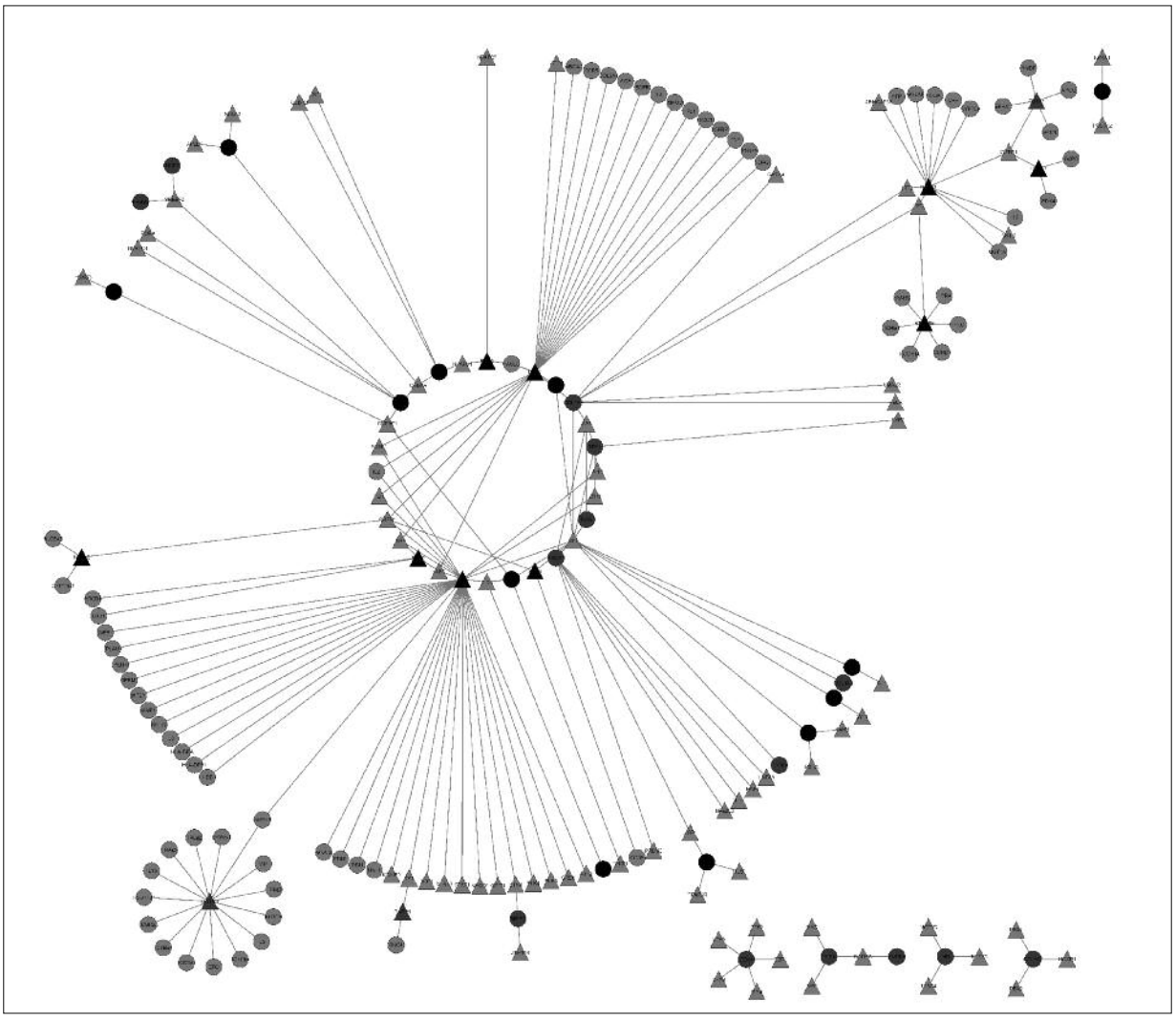

Figure 1. Transcriptional regulator network of 47 hormone-responsive genes. Triangles represent transcription factor (TF). Dots represent regulated target genes. The locations of 47 differentially expressed hormone-responsive genes are showed as colored nodes. Up-regulated genes are showed in red and down-regulated genes are showed in blue. Grey edge represents regulatory interactions.

the functional nodes identified can be classified into three different groups. The group response to hormone stimulus accounts for the biggest proportion. The second group is cell cycle related, and the third one is related with vascular development. Hence, we come to the conclusion that the DEGs, especially the hormone-responsive DEGs appear to exert principal influences on the progression of invasive ductal carcinoma.

Table III. Hub genes with degree > 4.2 in the transcriptional regulator network.

TF	Degree	TARGET	Degree
FOS	40	COL1A2	8
EGR1	23	HMOX1	8
GATA3	15	CDK1	5
NR3C1	14	PPARG	5
STAT5A	7	TFF1	5
FOXA1	5	–	–

Most studies have suggested that hub node gene has large effect on network and the related biological function[29,30]. In present study, with the aim of finding the important hub node genes in the development of IDC, we establish the Tnet and the PPI network on the basis of the hormone-responsive DEGs. We identify several hub node genes in the Tnet, including FOS, EGR1, GATA3, NR3C1, STAT5A, FOXA1, COL1A2, HMOX1, CDK1, PPARG, and TFF1. In the PPI network, these hub genes include PIK3R1, CDK1, PRKCD, ACTA1, NR3C1, CAV1, IRS1,

Table IV. Nodes with degree > 16.6 in the protein-protein interaction network.

Symbol	Degree	Symbol	Degree
PIK3R1	128	IRS2	35
CDK1	119	PPARG	35
PRKCD	102	CALR	33
ACTA1	90	NGFR	32
NR3C1	89	CASP6	30
CAV1	73	MMP9	30
IRS1	61	COL1A1	29
FOS	53	FGF2	27
STAT5A	52	FOXO1	25
ERBB2	47	AURKA	23
PRKDC	40	GHR	22
PARP1	40	MAP1B	22
FGFR3	39	TOP2A	20
CDH1	39	COL1A2	19
FHL2	38	EIF4EBP1	19
SPP1	19	MMP3	17
SORBS1	17	–	–

FOS, STAT5A, IRS2, PPARG, CALR, NGFR, CASP6, MMP9, COL1A1, FGF2, and FOXO1. KEGG pathway enrichment analysis demonstrated that many hub genes are involved in the tumor associated signaling pathways, such as Pathways in cancer, ECM-receptor interaction, Adherens junction, MAPK signaling pathway, Endometrial cancer, Prostate cancer, p53 signaling pathway, PPAR signaling pathway, ErbB signaling pathway, Toll-like receptor signaling pathway, which suggest the importance of hormone-responsive genes in progression of IDC.

In addition, we reviewed some literature and reconfirmed the function of some hub genes. Fos protooncogene (also known as c-fos), a transcription factor of the highest degree, was reported to be induced by estrogen in MCF-7 human breast cancer cells[31], which may be linked to both MAPK- and PI3K-dependent activation of the SRE through phosphorylation of Elk-1 and SRF[32]. Another report also claimed that c-fos could promote MCF-7 cells invasion[33]. Transcription factor EGR1 (early growth response 1) was suggested as a tumor suppressor gene and may function as important mediator of the biological effects induced by E2 and OHT (4-hydroxitamoxifen, an antagonist of ER) through GPER/EGFR/ERK signaling in breast cancer cells, which suggested EGR1 as a further target in novel therapeutic strategies[34,35]. With regard to target genes, COL1A2 (collagen type I±2) has been reported to contribute to proliferation and metastatic progression[36]. These results emphasize the important function of the hub genes and provide us more information about the carcinogenesis of breast.

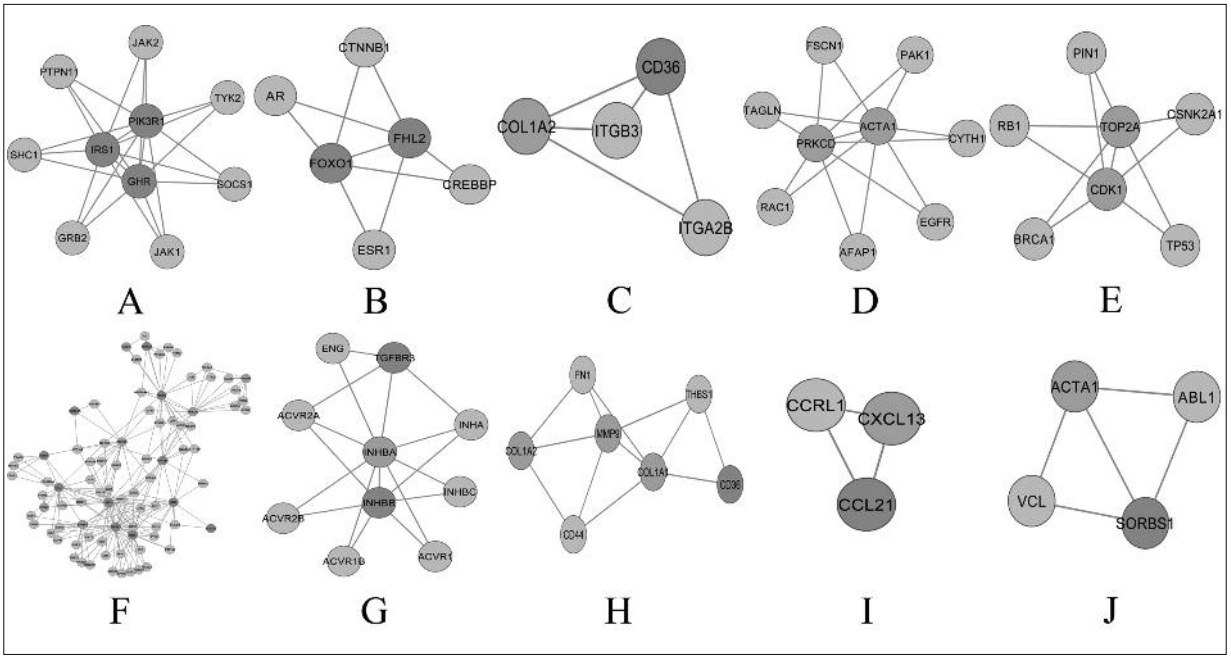

Figure 2. Ten functional modules in the Protein-protein interaction network. Dots represent corresponding genes of proteins. Grey edge represents protein-protein interactions. Up-regulated genes are showed in red. Down-regulated genes are showed in blue and other genes related with DEGs are showed in grey. A-J display 10 functional modules searched from the protein-protein interaction network.

Cancer is, in essence, a multiple genetic disease. Therefore, it is more important to evaluate the tight coupling modules in the network rather than only find the hub genes. In fact, we observe there are many modules in the PPI network. We select Module E as an example to illustrate the importance of these modules. Module E contains seven genes: BRCA1, CDK1, CSNK2A1, PIN1, RB1, TOP2A, and TP53, of which, CDK1, a member of cyclin-dependent protein kinase (CDK) family, is well documented as prognostic markers for multiple types of cancers, as well as breast cancer[37-44]. Some CDK inhibitors were used for the treatment of triple-negative breast cancers based on the MYC status, indicating CDK as a promising therapeutic target for tumors[45]. Topoisomerase (DNA) II alpha (TOP2A) play a key role in chromosome condensation, chromatid separation and the relief of torsional stress that occurs during DNA transcription and replication. The genomic locus, 17q12, which harbors the TOP2A, is the frequently amplified region in breast cancers[46]. It's noteworthy that well-known tumor suppressor gene RB1, TP53, as well as BRCA1 are genes that can interact with CDK1 and TOP2A, suggesting the important function of these genes in the development and progression of breast cancer. Pin1 (prolyl isomerase), also frequently overexpressed in human tumors which may be correlated with Cyclin D1[47,48] and has been shown to promote transformation of breast cells[49,50]. Moreover, it's essential for the proper activation of wtp53. On the contrary, it can also cooperate with mutp53 and support oncogenic function of mutp53[51]. CSNK2A1 (casein kinase 2, alpha 1 polypeptide), one of genes found in Module E, plays important role on regulation of p53 activity and modulation of breast cancer[52,53]. Taken together, we find every gene in Module E is associated with development and progression of cancer. They form the biological module structure through interaction with each other, from which we may explore novel therapies to treat breast cancers. We believe other modules are equally important, even if this requires further investigation.

Conclusions

Putting all these findings together, we identify some key genes, pathways and functional modules in progression of IDC and provide more information for mechanism study and targeted therapies of IDC.

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