Gene expression profile analysis of pancreatic cancer based on microarray data

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Abstract. The present study identified differentially-expressed genes (DEGs) between pancreatic cancer (PC) tissues and normal tissues, and assessed genetic factors associated with the pathogenesis of PC. The mRNA expression microarray dataset, GSE16515, containing 52 samples, including 16 paired tumor and normal tissue samples, and 20 tumor samples, was downloaded from Gene Expression Omnibus. Raw data were normalized and DEGs were identified. Subsequently, clustering was performed, protein-protein interaction networks were drawn, and functional and pathway enrichment analyses of the DEGs were performed. Copy number variations of DEGs were also identified. A total of 1,765 DEGs between PC and normal tissues were identified, including 1,312 upregulated and 453 downregulated DEGs. Upregulated DEGs were associated with the regulation of nucleocytoplasmic and intracellular transport, whereas downregulated DEGs were associated with the response to organic substances and hormone stimulus. The pancreatic cancer pathway was connected to three DEGs, namely transforming growth factor β1 (*TGFB1*), TGFβ receptor 1 (*TGFBR1*) and epidermal growth factor (*EGF*), which had 2, 3 and 5 CNVs, respectively. These results indicated the important roles of *TGFB1*, *TGFBR1* and *EGF* in the pathogenesis of PC. These genes may be potential therapeutic targets for the treatment of PC.

Introduction

Pancreatic cancer (PC; OMIM 260350) is a highly lethal disease, with an incidence rate that is constantly increasing (1). The 5-year survival rate of PC is <5% (2), and almost all patients with primary PC develop metastases.

Previous studies have indicated that PC has a complex genomic landscape with frequent copy number variations (CNVs)

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or copy number polymorphisms (CNPs) (3). Biankin *et al* (4) defined 16 significantly mutated genes [e.g., Kirsten rat sarcoma viral oncogene homolog (*KRAS*), tumor protein p53 (*TP53*), SMAD family member 4 (*SMAD4*) and transforming growth factor β receptor 2 (*TGFBR2*)] that were reaffirmed known mutations associated with PC. The commonly mutated genes, such as *KRAS* (74-100%), cyclin-dependent kinase inhibitor 2A (up to 98%), *TP53* (43-76%), erb-b2 receptor tyrosine kinase 2 (*ERBB2*; ~65%) and fragile histidine triad (~70%) have been found in PC (5-9). Among these genes, *KRAS* and *ERBB2* are proto-oncogenes, whereas the other genes are tumor suppressors (3). The progression of PC is correlated with the activation of oncogenes and the inactivation of tumor suppressor genes, as well as the deregulation of a number of signaling pathways, among which the epidermal growth factor receptor (*EGFR*), v-akt murine thymoma viral oncogene homolog 1 *(v-AKT1)* and nuclear factor of κ light polypeptide gene enhancer in B-cells 1 *(NFκB1)* pathways appear to be most relevant (10).

AKT1 is a central regulator of cell growth. *AKT1* has been shown to inhibit apoptosis and promote cell survival, thus contributing to the pathogenesis of cancer (11,12). Pei *et al* (13) showed that FK506-binding protein 51 (*FKBP51*) acted as a scaffolding protein for Akt and that it promoted the activation of Akt. The expression of *FKBP51* was downregulated in PC tissues. Decreased *FKBP51* expression resulted in the hyperphosphorylation of Akt, and then decreased the level of cell death in the PC tissues. Thus, Pei *et al* demonstrated *FKBP51* to be a negative regulator of the Akt pathway (13). Pei *et al* also released the mRNA expression microarray dataset, GSE16515, consisting of 36 pancreatic tumor and 16 normal tissue samples. Numerous studies have since been performed using this dataset (14-16). For example, using the GSE16515 dataset, Yang *et al* (14) screened the differentially-expressed genes (DEGs), such as TGF α (*TGFA)* and *EGF*, between PC tumor tissues and normal tissues, and selected the important single nucleotide polymorphisms (SNPs) of A/G and C/T in the DEGs. However, none of the studies based on the GSE16515 dataset performed an analysis of CNVs in the DEGs.

Using the GSE16515 dataset downloaded from Gene Expression Omnibus (GEO), the DEGs between PC tumor tissues and normal tissues were screened in the present study. Next, clustering analysis and construction of a protein-protein interaction (PPI) network of DEGs was performed. The underlying functions of these DEGs were investigated by functional and pathway enrichment analyses. Finally, the CNVs of these

DEGs were also analyzed. This will be beneficial for developing therapeutic strategies for patients with PC.

Materials and methods

mRNA microarray data. The mRNA expression microarray data from the GSE16515 dataset (6) was downloaded from GEO (http://www.ncbi.nlm.nih.gov/geo/), based on the platform of GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA, USA). GSE16515 was composed of 52 samples (from 34 males and 18 females). In total, 32 samples consisted of tumor and normal expression data, whereas 20 samples consisted of only tumor data. These samples were obtained during clinically indicated surgical procedures and consent was obtained for experimental purposes. The raw data and the probe annotation files were downloaded for further analysis. The microarray data of the GSE16515 dataset was analyzed following the procedures presented in Fig. 1.

Data preprocessing and DEG identification. The Robust Multiarray Average in Affy package of R (http://www.bioconductor.org/packages/release/bioc/html/affy.html), provided by Bioconductor project (17), was applied to process the raw microarray data. The processing included background correction, quantile normalization and probe summarization of expression values. The gene expression matrices were obtained for further analysis. Afterwards, the Linear Models for Microarray Data package was used to identify the gene signatures between the tumor and normal tissues, with significant differences indicated using a P-value of <0.05. Next, the Bonferroni correction (18) was applied to adjust the raw P-value for the false discovery rate (FDR) and to calculate the fold change (FC). In the present study, the cut-off criteria for the statistically significant DEGs were |log2FC| >1 and FDR <0.05.

Clustering analysis of DEGs. Based on the Euclidean distance between the expression profile of each DEG filtered from the samples, hierarchical clustering can be used to build a hierarchy of clusters of DEGs (19). The heatmap figure of the DEGs was drawn with the R package pheatmap (http://cran.r-project.org/web/packages/pheatmap/index.html) function. DEGs with the same signatures were clustered together, indicating the specificity of the DEGs.

Identification of PPIs of DEGs. Identification of protein complexes and functional modules from PPI networks is crucial to predict protein functions and to understand the principles of cellular organization (20). The Search Tool for the Retrieval of Interacting Genes (STRING; http://string-db.org/) database provides uniquely comprehensive coverage and ease of access for the prediction of interaction information (21). To better understand the interactions of the DEGs, the PPI network of their encoding products was predicted using the STRING database, with the reliability threshold of >0.9. Cytoscape software (http://cytoscape.org/), a standard tool for the integrated analysis and visualization of biological networks, was used to visualize the PPI network (22).

Functional enrichment analysis of DEGs. Gene Ontology (GO; http://www.geneontology.org/) analysis is an functional study
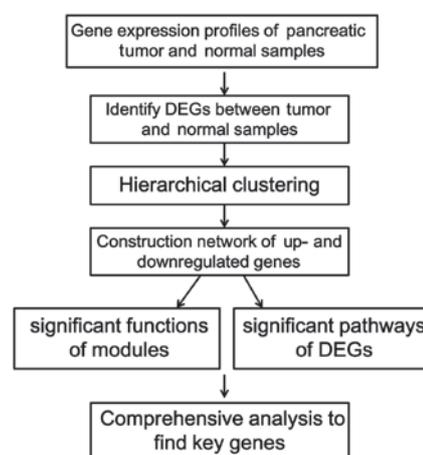

Figure 1. Flowchart of the analysis of the microarray data from the GSE16515 dataset. DEG, differentially-expressed gene.

method for large-scale transcriptomic or genomic data (23). In order to investigate the biofunctions of DEGs in tumor progression, the Database for Annotation, Visualization and Integrated Discovery (http://david.abcc.Ncifcrf.gov/), a high-throughput and integrated data-mining environment (24), was used to identify the enriched GO biological processes that the DEGs were associated with (FDR<0.05).

Pathway analysis of DEGs. The Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/pathway.html) pathway database provides information on how molecules or genes function (25). Pathway analysis of all the DEGs was performed using the KEGG database. The KEGG maps of biological functions associated with DEGs were obtained (P<0.05).

CNV analysis of DEGs. The Database of Genomic Variants (DGV; http://dgv.tcag.ca/) (26) was used to identify the CNVs in the DEGs, including deletions, insertions, duplications, complex multi-site variants and SNPs.

Results

Data processing and identification of DEGs. After the normalization, the DEGs between the tumor and normal tissues of the 52 samples were identified, with the cut-off criteria of |log2FC| >1 and FDR <0.05. A total of 1,765 DEGs were identified between the PC and normal tissues, of which 1,312 were upregulated and 453 were downregulated.

Hierarchical clustering of DEGs. Hierarchical clustering of the 1,765 DEGs is presented in Fig. 2. The LogFC values of the DEGs ranged from 6-fold downregulated and 6-fold upregulated. The majority of the DEGs were upregulated in the PC tumors compared with the normal tissues. The tumor samples and the normal control samples could easily be distinguished from the characteristics of the DEGs.

PPIs analysis of DEGs. To identify the PPIs and predict protein functions, PPI network analysis was performed using the STRING database (threshold >0.9). The resulting PPI network

Table I. Enriched GO biological processes of the DEGs in the protein-protein interaction networks.

Term and function	Count	Genes	P-value	FDR
Upregulated DEGs				
GO:0046822 - Regulation of nucleocytoplasmic transport	7	CDKN2A, TGFBR1, SMAD3, CDH1, TACC3, FLNA, TGFB1	1.72×10^{-6}	0.002848
GO:0032880 - Regulation of protein localization	9	TGFBR1, SMAD3, CDH1, CDH2, CASP1, TACC3, FLNA, TGFB1, IL1A	2.60×10^{-6}	0.004308
GO:0051222 - Positive regulation of protein transport	7	TGFBR1, SMAD3, CDH1, CASP1, FLNA, TGFB1, IL1A	4.05×10^{-6}	0.006710
GO:0032386 - Regulation of intracellular transport	7	CDKN2A, TGFBR1, SMAD3, CDH1, TACC3, FLNA, TGFB1	5.69×10^{-6}	0.009436
Downregulated DEGs				
GO:0043085 - Positive regulation of catalytic activity	13	ADCY1, PTGER3, CCKBR, C6, C5, LPAR3, EDNRB, PRKAR2B, PLCE1, CLPS, GNAS, EGF, PSMD6	1.72×10^{-5}	0.027131
GO:0010033 - Response to organic substances	15	TF, ADCY1, PNLIPRP1, GATM, PDE3B, EPHX1, CFTR, PDE3A, NPY1R, PRKAR2B, ABAT, ANGPT1, GNAS, SST, GNG7	2.20×10^{-5}	0.034801
GO:0009725 - Response to hormone stimuli	11	PRKAR2B, PNLIPRP1, ADCY1, GATM, PDE3B, GNAS, ANGPT1, CFTR, NPY1R, SST, GNG7	2.29×10^{-5}	0.036206
GO:0006575 - Cellular amino acid derivative metabolic processes	8	GSTA1, P4HB, CTH, GATM, GPX3, ABAT, GAMT, GNMT	2.78×10^{-5}	0.043955

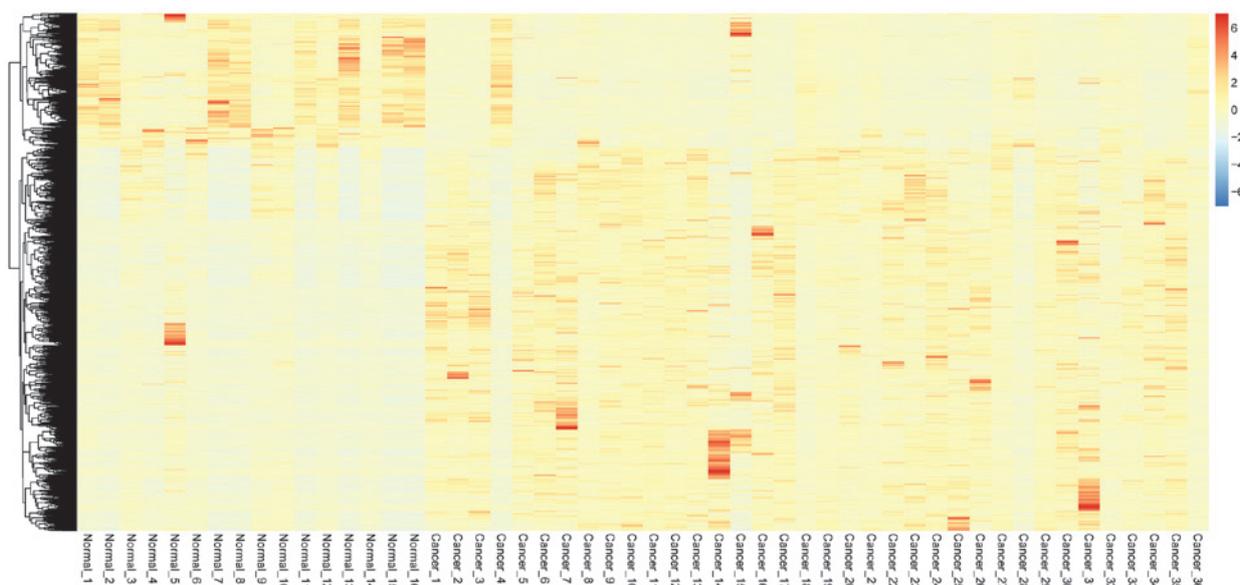FDR, false discovery rate; DEG, differentially-expressed genes; GO, gene ontology.

Figure 2. Heat map clustering of the differentially-expressed genes between two samples. The x-axis represents normal and tumor samples, and the y-axis represents genes. Blue (<0) indicates downregulation and orange (>0) indicates upregulation of gene expression in the pancreatic and normal tissues.

of upregulated DEGs connected to 92 nodes (proteins) through 171 PPIs, whereas the PPI network of the downregulated DEGs connected to 82 nodes through 83 PPIs (Fig 3).

Functional enrichment analysis of DEGs. To obtain the enriched GO biological processes of the DEGs in the PPI networks, GO functional enrichment analysis was performed for the up- and downregulated DEGs, respectively (FDR <0.05). The upregulated DEGs (including *TGFB1* and *TGFBR1*) were associated with significant biological processes, such as the regulation of nucleocytoplasmic transport, protein localization and intracellular transport (Table I), whereas the downregulated DEGs

A

B

Figure 3. Protein-protein interaction networks of (A) upregulated DEGs (orange) and (B) downregulated DEGs (blue). The nodes represent proteins and the lines between nodes represent interactions. DEG, differentially-expressed gene.

(e.g., *EGF*) were correlated with the biological processes of the positive regulation of catalytic activity, the response to organic substances and the response to hormone stimuli (Table I).

Pathway analysis of DEGs. The KEGG maps of biological functions associated with DEGs in the PPI networks were obtained (P<0.05). The results showed that only the pancreatic

Table II. Copy number variations in *TGFB1*, *TGFBR1* and *EGF* genes.

| First author, year	Gene	Variant ID	Subtype	(Ref.)
Xu *et al*, 2011	*TGFB1*	nsv911769	Loss	(27)
Shaikh *et al*, 2009		nsv521311	Insertion	(28)
Xu *et al*, 2011	*TGFBR1*	nsv893619	Insertion	(27)
		nsv893618	Insertion	
Wong *et al*, 2007		nsv831666	Insertion	(29)
Abecasis *et al*, 2012	*EGF*	esv2672203	Deletion	(30)
McKernan *et al*, 2009		esv2618042	Insertion	(31)
Conrad *et al*, 2010		esv22936	Loss	(32)
Mills *et al*, 2006		nsv290769	Loss	(33)
Kim *et al*, 2009		nsv820232	Loss	(34)
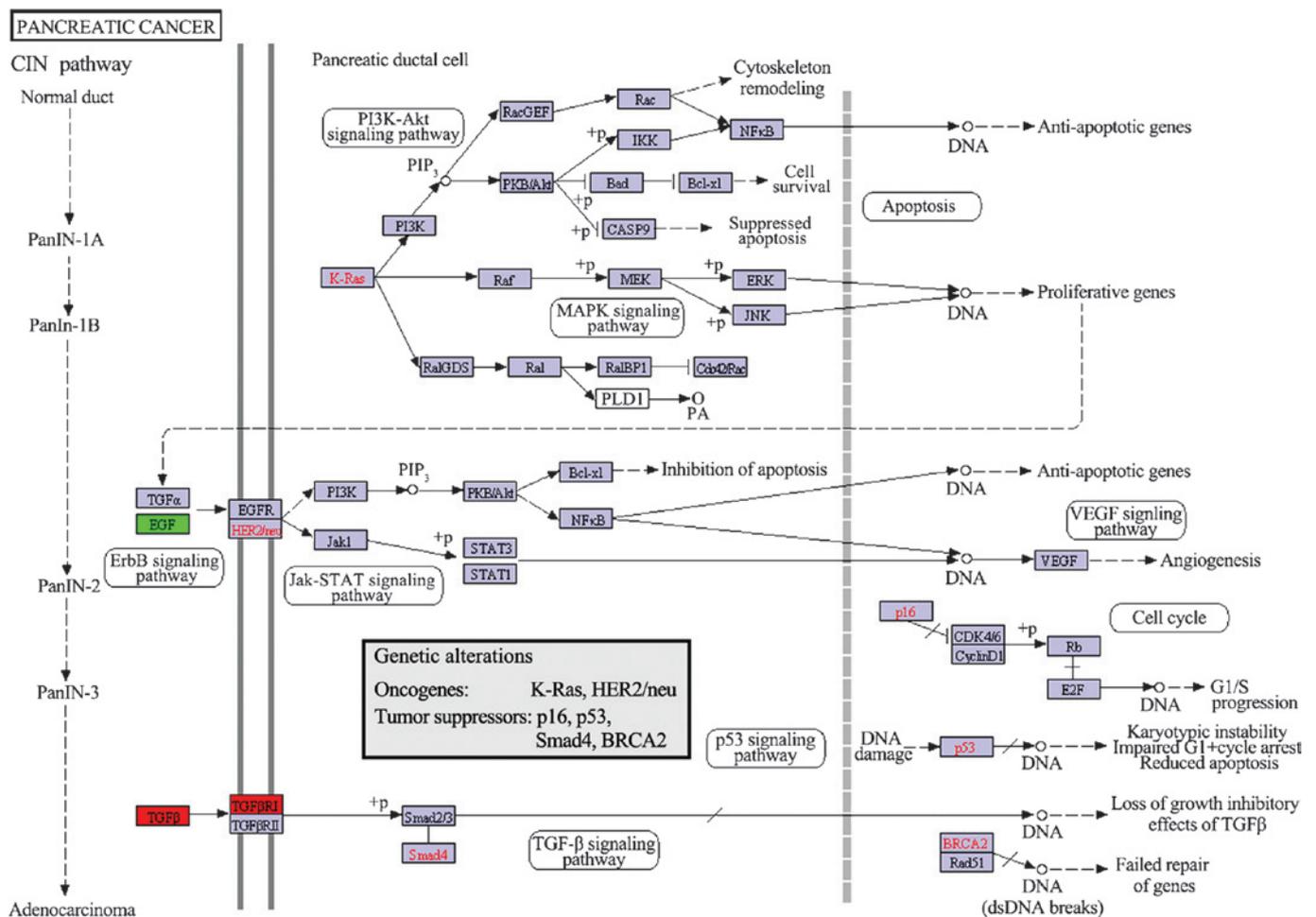
Figure 4. Molecular pathways in pancreatic cancer involving the DEGs in the protein-protein interaction networks. Red boxes represent upregulated DEGs and the green box represents a downregulated DEG. Red letters represent tumor suppressors or oncogenes that have been validated in previous studies. CIN, chromosomal instability; DEG, differentially-expressed gene; ds, double strand; PanIN, pancreatic intraepithelial neoplasia.

cancer pathway was associated with DEGs in the PPI networks, including upregulated *TGFB1* and *TGFBR1,* and downregulated *EGF* (Fig. 4).

CNV analysis of DEGs. The CNVs of the *TGFB1*, *TGFBR1* and *EGF* genes were further identified using the DGV. The identification of CNVs of DEGs included deletions, insertions, duplications and complex multi-site variants. Finally, 2, 3 and 5 CNVs, were identified in the *TGFB1*, *TGFBR1* and *EGF* genes, respectively (Table II) (27-34). In total, 1 of the CNVs of *TGFB1* was insertin; all 3 of the CNVs of *TGFBR1* were insertins; and 1 of the 5 CNVs of *EGF* was insertin.

Discussion

The early stages of PC are usually asymptomatic, and the majority of patients with PC are diagnosed at an advanced stage. The pathogenesis of PC is involved in a number of biological processes. Advanced studies of genetic factors have greatly improved our understanding of the pathogenesis of PC, which is associated with gene mutations, continuous changes to nuclei, loss of polarity and changes in cellular architecture (35).

In the present study, the DEGs between PC tumor tissues and normal tissues were systematically investigated. A total of 1,765 DEGs, including 1,312 upregulated and 453 downregulated DEGs, were identified. The majority of DEGs were upregulated in the tumor tissues. The upregulated DEGs (including *SMAD3*, *TGFB1* and *TGFBR1*) were associated with the regulation of nucleocytoplasmic and intracellular transport, and protein localization, whereas the downregulated DEGs (e.g., *EGF*) were associated with regulation of catalytic activity, and the responses to organic substances and hormone stimuli. All 4 of these DEGs were connected to the pancreatic cancer pathway. In addition, *TGFB1*, *TGFBR1* and *EGF* exhibited 2, 3 and 5 CNVs, respectively. These results proposed an important role for these DEGs in PC development.

CNVs or CNPs, such as deletions, insertions, duplications and complex multi-site variants, have been found in all humans and in other mammals (36). CNVs can be simple in structures like tandem duplication, or may be complex in the genome, such as in gains or losses of homologous sequences at multiple sites (37). Yang *et al* (14) screened the DEGs between PC tissues and normal tissues using the GSE16515 dataset, and selected the important SNPs of A/G and C/T in DEGs such as *TGFA* and *EGF*. In the present study, 5 CNVs of *EGF* were identified, including 3 losses, 1 deletion and 1 insertion. These results demonstrate that the CNVs of *EGF* may be significant in the pathogenesis of PC.

EGFR is required for *KRAS*-induced PC (38). Accordingly, the overexpression of *EGFR* and *TGFA* has been reported as an important molecular abnormality in human PCs (39). *EGF* and *TGFA* are essential molecules for the VEGF signaling pathway (Fig. 4). The inhibition of the vascular endothelial growth factor (VEGF) signaling pathway is beneficial for the suppression of tumor metastasis and invasion, such as required in PC (40). In the present study, it was found that the expression of *EGF* was downregulated in the PC tissues. Taken together, these results suggest the significant role of *EGF* in PC and indicate that *EGF* may be a novel target for the therapy of PC.

TGFB1 and *TGFBR1* were overexpressed in the PC tumors in the present study. The two genes are elements of the TGFB signaling pathway, which is a potent inhibitor of cell growth (41). There is growing evidence that members of the *TGFB* family are frequently mutated in cancer. CNVs, DNA segments that are ≥1 kb and present at variable copy number in comparison with a reference genome, affect the expression of genes, the variation and adaptation of phenotypes, and the pathogenesis of diseases, such as human immunodeficiency virus-1 infection, by disrupting genes and altering gene dosage in microdeletion or microduplication disorders (42,43). The present study identified 3 insertion CNVs in *TGFBR1*, and 1 insertion CNV and 1 loss CNV in *TGFB1*. Moreover, 3 of the CNVs in *TGFBR1* and *TGFB1* were located at the same segment as in a previous study (27).These indicate the vital roles of *TGFBR1* and *TGFB1* in PC development. The genes may be potential therapeutic targets for the treatment of PC.

In summary, 1,765 DEGs, including 1,312 upregulated (e.g.,*TGFB1* and *TGFBR1*) and 453 downregulated (e.g.,*EGF)* DEGS, were identified in the PC tissues compared with the normal tissues in the present study. The upregulated DEGs were associated with the regulation of nucleocytoplasmic and intracellular transport, whereas the downregulated DEGs were associated with the regulation of catalytic activity, and the response to organic substances and hormone stimuli. A pancreatic cancer pathway was connected to the DEGs of *TGFB1*, *TGFBR1* and *EGF*. In addition, *TGFB1*, *TGFBR1* and *EGF* exhibited 2, 3 and 5 CNVs, respectively. These results suggested the significance of the DEGs in PC. *TGFB1*, *TGFBR1* and *EGF* may be potential therapeutic targets for the treatment of PC. However, further clinical trials are required to validate these conclusions and hypotheses.

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