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Microarray based analysis of gene expression patterns in pancreatic neuroendocrine tumors

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Abstract. – OBJECTIVE: Pancreatic neuroendocrine tumors (PanNETs) are a small subgroup of tumors with a variety of biological behaviors.

MATERIALS AND METHODS: We sought to identify the specially expressed genes and characterize significant pathways in PanNETs compared with non-neoplastic samples. Gene expression profile GSE43795 was obtained from Gene Expression Omnibus database, which included 6 PanNETs and 5 non-neoplastic samples. The differentially expressed genes (DEGs) were identified using Limma package. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were used to enrich the functions and pathways of DEGs. Transcription factors (TFs) and tumor-associated genes (TAGs) were also identified.

Finally, a protein-protein interaction (PPI) network was constructed, and hub proteins and functional module were screened out.

RESULTS: Total of 821 DEGs (421 down-regulated, 400 up-regulated) were selected. GO and KEGG enrichment analyses showed that up-regulated DEGs were related to several pathways, including type 2 diabetes mellitus, Ca2+ signaling pathway, long-term potentiation, and long-term depression pathways. Down-regulated DEGs were enriched in several pathways, such as pancreatic secretion, protein digestion and absorption, and metabolic pathway. Interferon-stimulated gene protein 15 (ISG15), somatostatin (SST), and synaptosomal-associated protein 25 kDa (SNAP25) were identified as hub proteins.

CONCLUSIONS: The genes involved in type 2 diabetes mellitus pathway may play important roles in the development of PanNETs. SNAP25, SST, and ISG15 may be used as potential targets for treatment of PanNETs.

Key Words:
Pancreatic neuroendocrine tumors, Differentially expressed genes, Protein-protein interaction network.

Introduction

Pancreatic neuroendocrine tumors (PanNETs), usually known as islet cell tumors, islet cell carcinomas, or pancreatic endocrine tumors, are uncommon neuroendocrine tumors arising from endocrine cells of the pancreas[1,2]. According to world health organization (WHO) classification, PanNETs are distinguished as benign PanNETs, uncertain malignant potential tumors, low-grade and high-grade malignancy tumors[3]. Both the incidence and prevalence rate of PanNETs in all cases of pancreatic cancer are increasing, and the proportions are approximately 1.3% and 10%, respectively. Frequently, PanNETs are diagnosed at a late stage, and as a result, these patients have poor prognoses[4]. Although surgical resection is mainly used for the treatment of patients with metastatic or localized disease of PanNETs, systemic therapies for management of advanced tumors remain challenged due to limited options[5].

In order to investigate the pathogenesis of solid pseudopapillary neoplasm, Park et al[6] constructed a regulatory network between mRNA and microRNA through comparing the expression profiles of solid pseudopapillary neoplasm *vs.* non-neoplastic samples, PanNET vs. non-neoplastic samples, and pancreatic adenocarcinoma *vs.* non-neoplastic samples. The samples were from patients who had undergone pancreatic resection and were obtained immediately at the time of surgery. In PanNETs, several different cell surface markers such as somatostatin receptor subtype 2 (SSTR2) and glucagon-like peptide-1 receptor were found to be significantly expressed. However, the complete expression characteristics existing in PanNETs are not well described.

In this study, we identified the differentially expressed genes (DEGs) between PanNETs and

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non-neoplastic samples (no chronic pancreatitis or preneoplastic lesions) by comparing the expression profiles submitted by Park et al[6]. A protein-protein interaction (PPI) network was constructed. In addition, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genones (KEGG) pathway enrichment analyses were performed to provide more insight into the molecular mechanisms of PanNETs.

Materials and Methods

Microarray expression profile

Gene expression profiles data GSE43795 [6] including 31 samples were obtained from the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) data repository (http://www.nibi.nih.gov/geo/). A total of 11 samples were available in this study, of which 6 were PanNETs, and 5 were non-neoplastic tissues (platform: Illumina HumanHT-12 V4.0 expression beadchip).

Data Preprocessing and DEGs Analysis

The expression data were preprocessed using Limma[7], AFFY[8], and org.Hs.eg.db[9] package with Robust Multichip Average (RMA) algorithm[10], which includes background correction, quantile normalization, and final probe summarization. The average value was calculated as the expression value when several probes were corresponded to the same gene. The DEGs in PanNETs compared with non-neoplastic tissues were analyzed using Limma package[7]. Genes with $|\log_2 \text{fold change (FC)}| \geq 3$ and false discovery rate (FDR) corrected p-value < 0.01 were selected as DEGs.

Function Annotation and Pathway Enrichment Analysis

Transcription factors (TFs) were collected from TRANSFAC database[11], an integrated system for TFs, their DNA binding sites, and DNA-binding profiles. Tumor suppressor genes (TSGs) database[12] and tumor-associated genes (TAGs) database[13] were also screened to identify tumor suppressor genes and oncogenes. GO[14] was used to identify the enriched categories of cellular component (CC), molecular function (MF), and biological processes (BP), with FDR < 0.01. KEGG[15] pathway was used to understand the respective pathways of DEGs in cellular process, signal transduction, and biological pathways. The count number > 2 and FDR < 0.01 were used as cutoff criteria.

PPI Network Construction and Sub-Network Mining

Protein interaction pairs were downloaded in May 9, 2014 basing on Search Tool for the Retrieval of Interacting Genes (STRING)[16]. Interaction pairs contained at least one DEG with the combined score > 0.9 were input to construct the PPI network. Besides, a sub-network mining basing on BioNet[17] was performed to identify important PPI sub-networks. FDR-corrected $p < 0.001$ was set as the cutoff value.

Results

Screened DEGs

A total of 821 DEGs were identified in PanNETs compared with non-neoplastic tissues. Among them, 421 were down-regulated and 400 were up-regulated genes.

GO and KEGG Pathway Enrichment Analysis

As shown in Table I, KEGG pathway enrichment analysis suggested that up-regulated DEGs were mainly associated with type 2 diabetes mellitus, Ca^{2+} signaling pathway, ABC transporters, long-term potentiation, and long-term depression pathways. Down-regulated DEGs were primarily related to the following pathways: maturity onset diabetes of the young; secretion related pathway (e.g. pancreatic secretion and bile secretion); digestion, absorption and metabolism related pathways (e.g. glycine, serine, and threonine metabolisms).

The top 10 GO terms for up- and down-regulated DEGs were listed in Table II. The overrepresented GO terms for up-regulated DEGs were mainly associated with signal transduction and neural impulses related functions (e.g. multicellular organismal signaling, synaptic transmission, and transmission of nerve impulse) and transport and positioning related functions (e.g. single-organism transport and regulation of transport). Down-regulated DEGs were mainly enriched in digestion and metabolism related functions, such as digestion, sulfur compound metabolic process, cellular modified amino acid metabolic process.

Identification of TFs, TSGs and Oncogenes

A total of 20 TFs (7 up-regulated, 13 down-regulated), 11 oncogenes (3 up-regulated, 8 down-regulated), 26 TSGs (10 up-regulated, 16

Table I. The significantly enriched Kyoto Encyclopedia of Genes and Genones (KEGG) pathway by differentially expressed genes (DEGs).

	KEGG Pathway	Gene Counts	*p*-value
Up	Type 2 diabetes mellitus	5	0.0018266
	Ca^{2+} signaling pathway	9	0.0053779
	ABC transporters	4	0.0086306
	Long-term potentiation	5	0.0093588
	Long-term depression	5	0.0093588
Down	Pancreatic secretion	21	1.425E-12
	Protein digestion and absorption	16	1.704E-09
	Glycine, serine and threonine metabolism	8	3.594E-06
	Drug metabolism-cytochrome P450	11	1.07E-05
	Glutathione metabolism	9	1.589E-05
	Metabolism of xenobiotics by cytochrome P450	10	4.975E-05
	Fat digestion and absorption	7	0.0004267
	Maturity onset diabetes of the young	5	0.0008063
	Glycolysis/gluconeogenesis	7	0.0034124
	Proximal tubule bicarbonate reclamation	4	0.0047008
	Bile secretion	7	0.0055949
	Metabolic pathways	48	0.007581
	Arachidonic acid metabolism	6	0.0087252

Gene counts: number of DEGs enriched in KEGG pathways; $p < 0.01$ was considered to be significant.

down-regulated), and 9 other TAGs (3 up-regulated, 3 down-regulated) were identified, as shown in Table III. Furthermore, compared with Disease Ontology database, we found that some up-regulated genes were linked to movement disease, idiopathic generalized epilepsy, and osteosclerosis. Some down-regulated genes were concerned with microinvasive gastric cancer, prostate disease, stomach carcinoma, and lymphoblastic leukemia.

Table II. Gene Ontology (GO) analysis for differentially expressed genes (DEGs).

	GO ID	Term	Gene Counts	*p*-value
Up	GO:0035637	Multicellular organismal signaling	50	3.10E-12
	GO:0007268	Synaptic transmission	44	2.40E-11
	GO:0019226	Transmission of nerve impulse	47	3.86E-11
	GO:0007267	Cell-cell signaling	57	3.95E-10
	GO:0044765	Single-organism transport	106	7.88E-09
	GO:0006810	Transport	120	1.49E-08
	GO:0051234	Establishment of localization	121	1.93E-08
	GO:0050877	Neurological system process	57	4.35E-08
	GO:0051179	Localization	136	3.33E-07
	GO:0051049	regulation of transport	49	3.538E-07
Down	GO:0007586	Digestion	26	0
	GO:0006790	Sulfur compound metabolic process	24	4.09E-09
	GO:0006575	Cellular modified amino acid metabolic process	18	9.34E-07
	GO:0044699	Single-organism process	299	1.25E-06
	GO:0006749	Glutathione metabolic process	9	1.66E-06
	GO:0044281	Small molecule metabolic process	96	3.45E-06
	GO:0006805	Xenobiotic metabolic process	15	4.89E-06
	GO:0071466	Cellular response to xenobiotic stimulus	15	5.292E-06
	GO:0044710	Single-organism metabolic process	110	6.635E-06
	GO:0009410	Response to xenobiotic stimulus	15	6.678E-06

Gene counts: number of DEGs involved in GO terms; $p < 0.01$ was considered to be significantly different.

Table III. Differentially expressed transcription factors (TFs) and tumor associated genes (TAGs) in pancreatic neuroendocrine tumors.

	TFs	Oncogenes	TSGs	Other TAGs
Up	ZFP37, SMAD9, RFX2, MYT1, MEIS3, KLF12, GTF2H2,	TPD52, MPL, DUSP26;	PHLPP2, NEURL, KL, ISG15, IGFBP3, HPGD, HOPX, GADD45GIP1, CHD5, CEACAM1;	MAP1A, DHDH, ABCB1,
Down	TEAD4, SIX5, PKNOX2, PBX1, ONECUT1, NR5A2, NKX2-5, LMO3, KLF15, KCNIP3, IRX5, HHEX, GATA4,	VAV3, SPHK1, PBX1, MYC, LCN2, GFI1, GATA4, CXCL1;	STEAP3, SRPX, SFRP5, SERPINI2, SEMA3B, PPP1R1B, PLCE1, ONECUT1, MUC1, MT1G, GPC3, GNMT, GAS1, DEFB1, CEBPA, BTG2;	TPD52L1, SLC43A1, CHRM3

Table IV. The KEGG pathway enrichment analysis of genes involved in the PPI sub-network.

KEGG Pathway	Gene Counts	*p*-value	Gene
SNARE interactions in vesicular transport	5	3.59E-10	SNAP25, VAMP2, STX3, STX1A, STX2
Vasopressin-regulated water reabsorption	2	0.001494267	VAMP2, PRKACA
Vibrio cholerae infection	2	0.002244964	CFTR, PRKACA
Bile secretion	2	0.003853445	CFTR, PRKACA
Gastric acid secretion	2	0.004179809	CFTR, PRKACA
Salivary secretion	2	0.005998157	VAMP2, PRKACA

Gene counts: the number of genes enriched in KEGG pathway. $p < 0.001$ was considered to be significant.

PPI Network Analysis

In order to identify potential target genes and functional modules in PanNETs, a PPI network was constructed (Figure 1). Ten nodes with high degree were v-myc avian myelocytomatosis viral oncogene homolog (MYC, degree = 93), epidermal growth factor (EGF, degree = 42), interferon-stimulated gene protein 15 (ISG15, degree = 34), albumin (ALB, degree = 29), topoisomerase (DNA) II alpha 170 kDa (TOP2A, degree = 26), somatostatin (SST, degree = 24), WD repeat domain 5 (WDR5, degree = 21), glutathione S-transferase alpha 2 (GSTA2, degree = 20), chemokine (C-X-C motif) ligand 11 (CXCL11, degree = 19), and ras-related C3 botulinum toxin substrate 3 (RAC3, degree = 19), respectively. Among them, ISG15, TOP2A, and RAC3 were up-regulated, and the other 7 genes were down-regulated.

Moreover, a PPI sub-network with 10 nodes was found using BioNET. As shown in Figure 2, synaptosomal-associated protein 25 kDa (SNAP25, degree = 5) was the hub protein. The KEGG pathway enrichment analysis showed that these genes were associated with secretion related pathways (e.g. bile secretion, gastric acid secretion, and salivary secretion), SNARE interactions in vesicular transport, and vasopressin-regulated water reabsorption pathways (Table IV).

Discussion

In this study, a total of 821 DEGs (421 down-regulated, 400 up-regulated) were selected in PanNETs compared with non-neoplastic tissues. We demonstrated that up-regulated DEGs were related to several pathways, including type 2 diabetes mellitus, Ca^{2+} signaling pathway, long-term potentiation, and long-term depression pathways. Down-regulated DEGs were enriched in several pathways, such as pancreatic secretion, protein digestion and absorption, and metabolic pathway. In addition, SNAP25, SST, and ISG15 were hub proteins.

Type 2 diabetes is closely associated with pancreatic cancer. An article reviewed previous studies about the relationship between diabetes and pancreatic cancer. The result showed that evidence supported 2 hypotheses: one is that diabetes is caused by pancreatic cancer and another

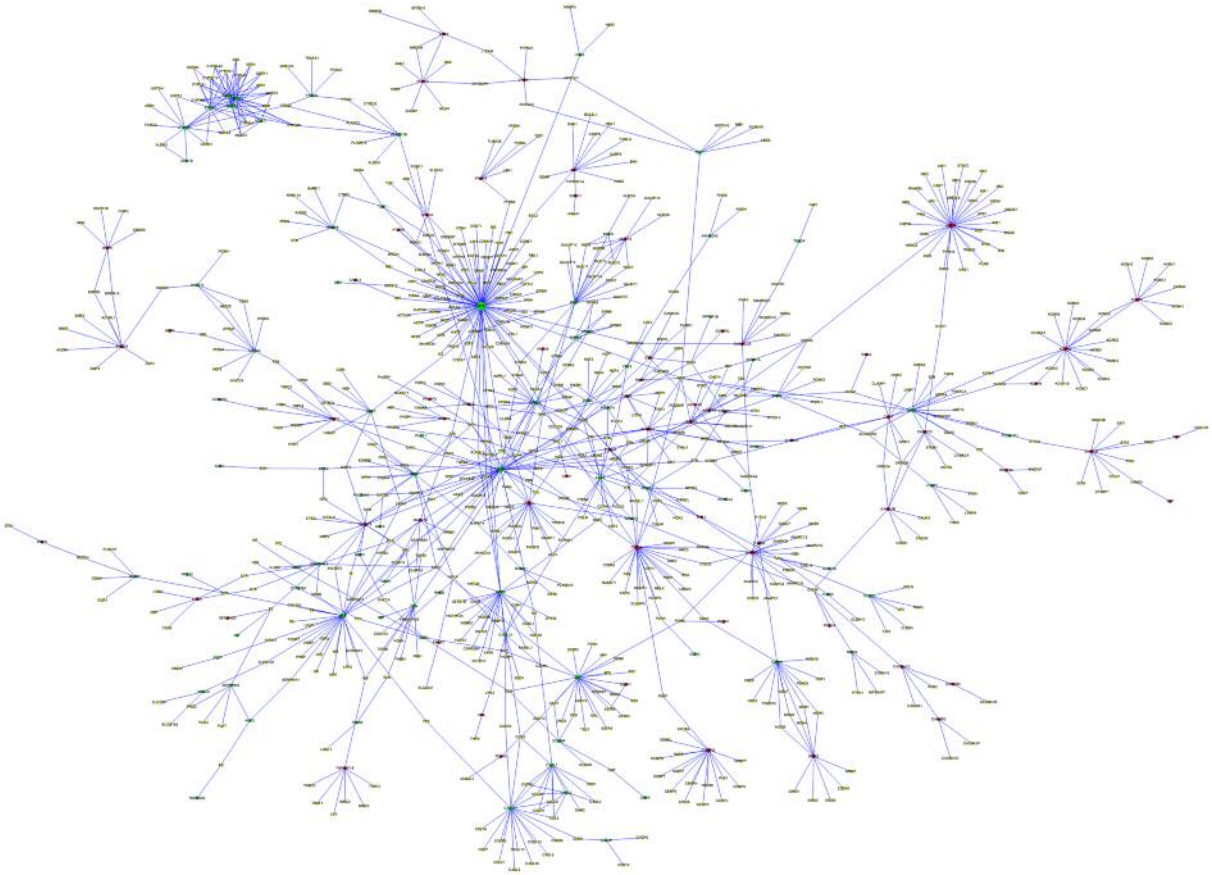

Figure 1. Protein-protein interaction (PPI) network of differentially expressed genes (DEGs). Red circles represent up-regulated DEGs; green circles represent down-regulated DEGs; and yellow circle represents non-DEGs.

one is that diabetes promotes the development of pancreatic cancer[18]. Subsequently, a meta-analysis of 36 studies[19] was performed and the result demonstrated that type 2 diabetes was causally associated with pancreatic cancer. Moreover, abnormal glucagon signaling could lead to hyperglycemia of type 2 diabetes. It has been shown that glucagon signaling inhibition is closely associated with hyperglucagonemia and hyperplasia of pancreatic α cells in mice. Yu et al[20] found that a patient with an inactive mutation of glucagon receptor also showed hyperglucagonemia and hyperplasia of pancreatic α cells, but further developed PanNETs. In the present study, we found that some up-regulated genes were enriched in type 2 diabetes mellitus by KEGG enrichment pathway analysis in PanNETs compared with non-neoplastic tissues. These genes involved in the type 2 diabetes mellitus pathway may play important roles in the development of PanNETs.

Moreover, SNAP25 was identified as a hub protein in the present study. SNAP25 functions as a complex (SNARE) along with syntaxin (STX) and synaptobrevin (VAMP), and this process is triggered by micromolar concentrations of Ca^{2+}. In pancreatic islets, SNAP25 interacts with Ca^{2+}-binding proteins, such as secretagogin and synaptotagmin I. When synaptotagmin I binds to SNARE complex, Ca^{2+} induces a conformational change in the prebound synaptotagmin and then leads to a variety of events that opens the fusion pore[21]. It has been reported that SNAP25 is completely expressed and is used as a neuroendocrine marker in medullary carcinoma of thyroid epithelial tumor and in large bowel NETs[22-24]. Besides, SNAP-25 and VAMP have been reported to be expressed in pancreatic acinar cells[25]. Consistently, SNAP25 was up-regulated in our study. Thus, SNAP25 may also be a neuroendocrine marker for PanNETs. However, more studies are needed to confirm this hypothesis.

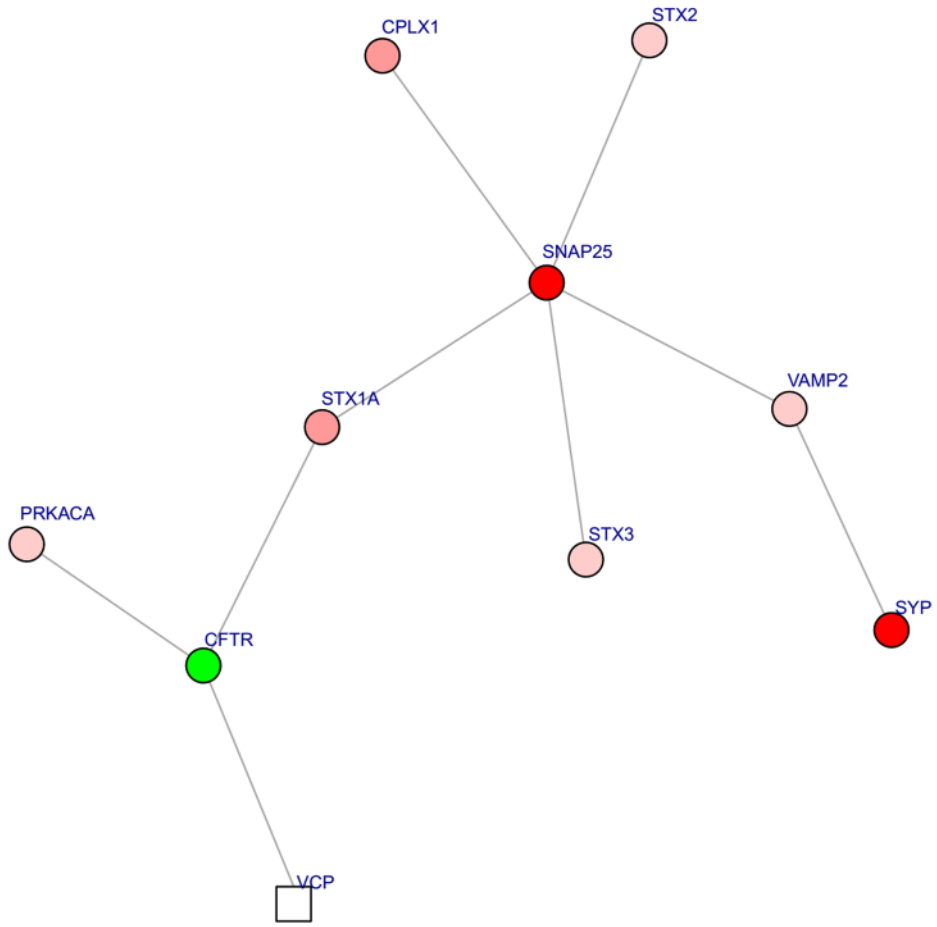

Figure 2. The core sub-network in PPI network. Red circles represent up-regulated genes; green circles represent down-regulated genes; white and pink circles represent non-differentially expressed genes. Squares represent low contribution to the PPI sub-network.

Besides, SST, also termed as somatotropin release inhibiting factor (SRIF), plays an important regulatory role in endocrine and exocrine secretion and affects several hormones release such as glucagon, insulin, and thyroid-stimulating hormone[26]. It serves as a neuromodulator and neurotransmitter in central nervous system. SST inhibits hormones release through hindering Ca^{2+} entry into hormone-sensitive cells[27]. The effects of SST are performed by association with G protein-coupled membrane receptors (termed SSTR1-5)[28]. These five SSTRs regulate various intracellular signaling pathways including the adenylyl cyclase-cAMP-protein kinase A system, Ca^{2+}, K^+ channels and serine/threonine and tyrosine phosphatases[29]. In 90% human pancreatic adenocarcinomas, SSTR2 expression is lost, and stable transfection of SSTR2 can inhibit cell proliferation, tumorigenicity, and metastasis in human pancreatic cancer cells[30,31]. Previously, it has been demonstrated[32] that SST can inhibit cell growth of pancreatic cancers which express SSTR2. Moreover, it has been suggested[33] that chronic administration of SST analogs causes growth inhibition of a variety of tumors in animals, such as pancreatic, prostatic, chondrosarcomas, breast, and pituitary cancers. In the current work, SST was significantly down-regulated in PanNETs and it was a hub protein with degree of 24 in the PPI network. Combined SST (analogue) and its receptors may be taken into considered in the future treatment of PanNETs.

In addition, ISG15 was defined as one of the TAGs in the present study and was one of the hub proteins in the PPI network. It is a small ubiquitin-like protein and conjugated to target proteins via an enzymatic cascade. Recent evidences have suggested that it may be involved in several pathologies such as innate immunity and cancer[34,35]. In cancer cells, deletion of ISG15 in-

creases the levels of polyubiquitinated proteins, which suggests that there is an antagonistic relationship between ISG15 expression and ubiquitin-mediated proteinturnover[36]. Increased expression of ISG15 has been found in several cancers[37,38] such as melanoma, endometrial cancer, breast cancer, prostate cancer, cervical cancer, and pancreatic carcinoma. Besides, Ina et al[39] demonstrated that ISG15 was the gene that contributed to the sensitivity of gemcitabine, which is the standard chemotherapy agent for treatment of pancreatic cancer. Gemcitabine resistance could be reversed through inhibiting ISG15 transcription by siRNA in gemcitabine-resistant cells. In our study, ISG15 was also significantly up-regulated in PanNETs. Therefore, ISG15 might have a possibility to be target of PanNETs treatment.

Conclusions

The gene expression profiles are changed in the development and progression of PNETs. SNAP25, SST, ISG15, and the genes involved in type 2 diabetes mellitus pathway may play important roles in the development of PanNETs. These genes may be used as potential targets in the treatment of PanNETs. However, further studies should be carried out to confirm these hypotheses.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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