

Bioinformatics analysis of transcription profiling of solid pseudopapillary neoplasm of the pancreas

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Abstract. Solid pseudopapillary neoplasm (SPN) of the pancreas is a low-grade malignant neoplasm that accounts for ~5% of cystic pancreatic tumors and ~0.9-2.7% of exocrine pancreatic tumors. The transcription profiling data (GSE43795) of 14 SPN and 6 control samples were downloaded from the Gene Expression Omnibus (GEO) database. Using the Limma package, Student's *t*-tests were performed to identify differentially expressed genes (DEGs) between SPN and control samples [with the following criterion: False discovery rate (FDR) < 0.01 and log₂ fold-change (FC) ≥ 3]. Pathway and functional enrichment analyses were performed to investigate the biological processes that the DEGs were involved in. Protein-protein interaction (PPI) network and sub-network analyses were conducted to comprehensively understand the interactions between DEGs. The screened DEGs were further annotated according to information relating to transcription factors and tumor associated genes (TAGs). A total of 710 upregulated and 710 downregulated DEGs were observed, including 74 transcriptional factors and

124 TAGs. Membrane metallo-endopeptidase (MME), matrix metalloproteinase (MMP)-2 and MMP-9 were also identified as key TAGs. Following PPI network analysis, hub nodes of epidermal growth factor receptor (EGFR), proto-oncogene tyrosine protein kinase Fyn (FYN), c-JUN (JUN), glucagon (GCG), c-Myc (MYC) and CD44 were identified, the majority of which participate in the epidermal growth factor receptor (ErbB) and gonadotropin-releasing hormone (GnRH) signaling pathways. A sub-network involving 70 gene nodes was also identified, with EGFR as the central gene. MME, MMP-2 and MMP-9 contribute to proliferative diabetic retinopathy and also involved in SPN. The genes EGFR, FYN, JUN, GCG, MYC and CD44 may therefore be key genes in SPN, and the ErbB and GnRH signaling pathways may be an important contributor to SPN progression.

Introduction

Solid pseudopapillary neoplasm (SPN) of the pancreas is a low-grade malignant neoplasm with circumscribed, variegated, hemorrhagic, solid and cystic features (1). SPN was first described by Frantz in 1959 (2), and in 2010 the World Health Organization defined the cancer as solid pseudopapillary neoplasm of the pancreas (3). SPN accounts for ~5% of cystic pancreatic tumors and ~1-6% of all exocrine pancreatic tumors (4). Despite primarily occurring in younger women, patients with SPN have been reported to range from 2-85 years old (5). SPN is currently treated by complete surgical excision, and diagnosed either by imaging, using electron microscopy, or histology, using immunohistochemistry. However, the exact molecular pathology and pathogenesis of SPN remains unclear (6).

SPN pathogenesis has been investigated extensively. Activation of the Wnt-β-catenin signal pathway, associated with mutations of exon 3 in the β-catenin gene, CTNNB1, may be involved in the tumorigenesis of SPN (7-9). β-catenin acts as a transcriptional activator in conjunction with T cell factor and lymphoid enhancer factor in the Wnt-β-catenin pathway, inducing the expression of target genes, and these may be useful diagnostic molecular markers (10). Kang *et al* (11) demonstrated that expression of the Wnt-β-catenin signaling pathway targets genes for matrix metalloproteinase (MMP)-7,

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Abbreviations: SPN, solid pseudopapillary neoplasm; DEG, differentially expressed gene; PPI, protein-protein interaction; TAG, tumor associated gene; GEO, Gene Expression Omnibus; KEGG, Kyoto Encyclopedia of Genes and Genomes; DAVID, The Database for Annotation, Visualization and Integrated Discovery; STRING, Search Tool for the Retrieval of Interacting Gene/Proteins; MMP, matrix metalloproteinase; ECM, extracellular matrix; PKC, protein kinase C

Key words: solid pseudopapillary neoplasm, differentially expressed genes, functional enrichment analysis, protein-protein interaction, tumor associated genes

cyclin-D1 and c-Myc, and may result in an unpredictable clinical course in SPN. β -catenin is also involved in cell-cell adhesion, helping E-cadherin to link to the cytoskeleton (12). Silencing of E-cadherin mutations and nuclear translocation of β -catenin following activation of mutations results in loss of adherens junctions, and this same loss is commonly observed in patients with SPN (13).

However, little is known about SPN besides the activation of the Wnt- β -catenin signaling pathway. In order to identify the molecular pathogenesis of SPN, microarray data were downloaded and analyzed to identify differentially expressed genes (DEGs) between SPN and non-neoplastic pancreatic tissues. Significantly enriched pathways and functions were also screened, followed by the functional annotation of DEGs based on transcription factor and tumor-associated gene databases. Resultantly, a protein-protein interaction (PPI) network of DEGs was constructed and visualized.

Materials and methods

Obtaining and preprocessing of mRNA expression profile data. The mRNA expression profiles of SPN and non-neoplastic pancreatic tissues were obtained from the National Center of Biotechnology Information Gene Expression Omnibus (GEO, <https://www.ncbi.nlm.nih.gov/geo/>) database. The access number was GSE43795 (14), and datasets from 14 SPN samples and 6 control samples were used for further analysis. The platform used was Illumina Human HT-12 V4.0 Expression BeadChip (Illumina, Inc., San Diego, CA, USA). Original data were preprocessed with the Limma package (version 3.2.2; <http://www.bioconductor.org/packages/release/bioc/html/limma.html>) (15), AFFY package (version 1.32.0; <http://www.bioconductor.org/packages/release/bioc/html/affy.html>) (16) and the org.Hs.eg.db package using Bioconductor software (version 2.14; Fred Hutchinson Cancer Research Center, Seattle, WA, USA). Preprocessing of the data included background correction (17), quantile normalization and probe summarization. The expression matrix was then obtained, with each row representing the expression of a gene, and each column a sample.

DEGs screening. Bayesian analysis was performed using the Limma package (15), to identify DEGs between SPN and control samples. $FDR < 0.01$ and $\log_2 FC \geq 3$ were used as the thresholds.

Enrichment analysis of DEGs. To study DEGs at functional level, gene ontology (GO, <http://www.geneontology.org>) functional enrichment analysis (18) and Kyoto Encyclopedia of Gene and Genomes (KEGG; <http://www.genome.jp/kegg/pathway.html>) pathway enrichment (19) were performed using the Database for Annotation Visualization and Integrated Discovery (DAVID; version 6.7) software, an online biological tool (20). GO is a collection of controlled vocabularies, and only the biological process functions were enriched. $P < 0.01$ was set as the cut-off criterion for enrichment analysis.

Gene functional annotation analysis. Functional annotation analysis of genes is an important task, as it demonstrates associations between genes and biological pathways (21).

According to the information on transcription factors provided by TRANSFAC (version 11.2), the screened DEGs were further annotated. In order to investigate the molecular mechanism of SPN, all known oncogenes and tumor suppressor genes were extracted, based on the Tumor Associated Genes (TAG) database (version 3.07) (22), the Tumor Suppressor Gene database (version 2.0) (22) and the work of Zhao *et al* (23).

PPI network construction and sub-network detection. PPI network analysis is necessary to comprehensively understand the intracellular process. The Search Tool for the Retrieval of Interacting Gene/Proteins (STRING) database (24) has been widely used to construct PPI networks. To begin, PPI data (verified through experiments, text mining and co-expression analysis) were downloaded (2014.05.09) from STRING (version 10.0; <http://string-db.org/>). All DEGs were mapped to this dataset and a threshold of combined score ≥ 0.9 was applied to screen the interaction pairs. Finally, selected pairs were visualized using Cytoscape software (version 3.2.0; National Institute of General Medical Sciences, Bethesda, MD, USA).

The identification of significantly differentially expressed sub-networks within a large network is the primary task when a PPI network is constructed. The BioNet package (version 2.1) (25) was employed for sub-network analysis, and $FDR < 0.0001$ was set as the cut-off criterion. KEGG enrichment analysis was also performed at the sub-network level.

Results

DEG screening. Bayesian analysis was performed on the mRNA expression profile data with the criteria $FDR < 0.01$ and $\log_2 FC \geq 3$. Based on these criteria a total of 1,420 DEGs were screened out, among which 710 DEGs corresponding to 751 transcripts were upregulated and 710 DEGs corresponding to 746 transcripts were downregulated.

Enrichment analysis of DEGs. KEGG pathway enrichment analysis indicated that the 710 upregulated DEGs were enriched in 10 pathways, including pancreatic secretion, maturity onset diabetes of the young, protein digestion and absorption, while the 710 downregulated DEGs were enriched in 17 pathways, including the Wnt signaling pathway, melanogenesis, axon guidance, protein digestion and absorption ($P < 0.01$). The top ten pathways are listed in Table I.

GO functional enrichment analysis demonstrated that the 710 upregulated DEGs were enriched in 47 functions, including digestion, secretion and the cellular response to zinc ions, and the 710 downregulated DEGs were enriched in 88 pathways, including nervous system development, cell differentiation and neuron differentiation ($P < 0.01$). The top ten pathways are listed in Table II.

Gene functional annotation analysis. To investigate the molecular mechanisms of SPN, the function of DEGs as transcriptional factors and TAGs were also analyzed. A total of 74 DEGs were transcriptional factors, among which 31 were downregulated and 43 were upregulated; and 124 DEGs were TAGs, among which 73 were downregulated and 51 were upregulated (Table III). Additionally, through comparison with

Table I. KEGG pathway analysis of differentially expressed genes.

Pattern	KEGG pathway	Gene counts	P-value
Down	Pancreatic secretion	28	2.55E-15
	Maturity onset diabetes of the young	12	1.97E-10
	Protein digestion and absorption	19	2.12E-09
	Drug metabolism-cytochrome P450	14	3.98E-06
	Proximal tubule bicarbonate reclamation	7	4.99E-05
	Metabolism of xenobiotics by cytochrome P450	12	7.35E-05
	Fat digestion and absorption	8	9.77E-04
	Glutathione metabolism	8	1.72E-03
	Tyrosine metabolism	7	2.26E-03
	Starch and sucrose metabolism	8	2.84E-03
Up	Wnt signaling pathway	15	3.92E-04
	Melanogenesis	11	1.16E-03
	Axon guidance	12	2.77E-03
	Protein digestion and absorption	9	2.82E-03
	Leukocyte transendothelial migration	11	3.54E-03
	Cell adhesion molecules (CAMs)	12	3.56E-03
	Basal cell carcinoma	7	3.84E-03
	Pathways in cancer	22	4.23E-03
	Arrhythmogenic right ventricular cardiomyopathy	8	5.66E-03
	Tight junction	11	9.33E-03

KEGG, Kyoto Encyclopedia of Genes and Genomes.

Table II. Significantly enriched biological process function of differentially expressed genes.

Pattern	GO ID	Term	Gene counts	P-value
Down	GO:0007586	Digestion	31	1.11E-16
	GO:0046903	Secretion	72	6.84E-10
	GO:0071294	Cellular response to zinc ion	8	9.69E-10
	GO:0031018	Endocrine pancreas development	15	1.10E-09
	GO:0035270	Endocrine system development	22	1.16E-08
	GO:0001525	Angiogenesis	39	4.71E-08
	GO:0010038	Response to metal ion	28	9.86E-08
	GO:0030001	Metal ion transport	48	4.77E-07
	GO:0042593	Glucose homeostasis	20	9.07E-07
	GO:0071248	Cellular response to metal ion	15	1.11E-06
Up	GO:0007399	Nervous system development	119	5.82E-10
	GO:0030154	Cell differentiation	168	8.72E-10
	GO:0030182	Neuron differentiation	79	3.39E-09
	GO:0001501	Skeletal system development	42	5.94E-09
	GO:0043392	Negative regulation of DNA binding	9	1.01E-05
	GO:0046189	Phenol-containing compound biosynthetic process	7	6.87E-05
	GO:0060412	Ventricular septum morphogenesis	7	8.76E-05
	GO:0007268	Synaptic transmission	46	9.26E-05
	GO:0002720	Positive regulation of cytokine production involved in immune response	6	1.02E-04
	GO:0007155	Cell adhesion	59	3.00E-04

GO, Gene Ontology; GO ID, GO identification.

Table III. Functional statistics of differentially expressed genes between solid pseudopapillary neoplasm and control samples.

Pattern	TF counts	TF genes	TAG counts	TAG genes
Down	31	<i>CDX2, EHF, ELF3, FOSB, FOXA2, FOXA3, FOXC1, FOXQ1, GATA4, HEYL, HHEX, HNF4G, INSM1, ISL1, KCNIP3, KLF5, LMO3, MEIS1, NKX2-2, NKX2-5, NR4A2, NR5A2, ONECUT1, PAX6, PBX3, PDX1, PKNOX2, PLAGL1, SOX9, TEAD4, XBP1</i>	73	Oncogene: <i>CD24, CXCL1, EGFR, ELF3, ERBB3, FGFR1, FGFR3, GATA4, GFII, GPX2, JUN, LCN2, MEIS1, MYC, SPHK1</i> Tumor suppressor: <i>WNK2, VILI1, UCHL1, TPM1, TFPI2, SYT13, STEAP3, SRPX, SIK1, SFRP5, SERPINI2, RAP1GAP, RAB25, PTPRK, PRKCDBP, PLK2, PLAGL1, PDX1, PDGFRL, PAX6, ONECUT1, NRCAM, MUC1, MTUS1, MT1G, MEG3, LPL, KLF5, KLF10, ID4, GNMT, GAS1, FOXC1, FOXA2, ERRF11, EPHA1, ENC1, EHF, DEFB1, DAPK1, CLDN23, CEBPA, CDH1, C2orf40, BTG2, BMP2, BIN1, ADAMTS9</i> Other: <i>TACC2, SLC43A1, RRAS2, PBX3, NR4A2, MAP3K5, GRB7, CHRM3, CDX2, CD44</i>
Up	43	<i>TWIST2, TFAP2C, TCF7, TBX3, T, SOX11, SIM2, SHOX2, RUVBL1</i> <i>RUNX2, REST, PRDM1, PITX2, PGR, NR0B1, NFAT5, MAFG, MAF, LEF1, KLF12, HOXC9, HOXC8, HOXC6, HOXC5, HOXC4, HOXB8, HOXB7, HOXB3, HEY2, HEY1, HAND2, HAND1, GTF2H2, GLI2</i> <i>GATA1, FUBP1, ETV5, ESRRG, EMX2, DR1, DBP, BARX2, AR</i>	51	Oncogene: <i>RUNX2, NRAS, NOV, NET1, MME, MLLT11, MAP3K8, MAFG, LAMC2, GNAI2, FYN, FGF20</i> Tumor Suppressor: <i>ZBTB7C, WNT5A, WIF1, TWIST2, TMEM127, TMEFF2, THSD1, SOX11, RASL10B, PTPRG, PRDM1, PPP1R1B, MIR185, MCPH1, LSAMP, ISG15, HPGD, GLIPR1, FANCD2, DKK3, CSMD1, CNTNAP2, CDKN2D, CDH11, CABLES2, C10orf90, BIK, AXIN2, ARHGAP29, ARHGAP20</i> Other: <i>WNT2B, TPH1, TPD52L1, TFAP2C, PITPNA, OGG1, MCC, MAF, HOXC6</i>

TF, transcriptional factor; TA, tumor associated genes; TAG_ONCO, oncogene of tumor associated genes; TAG_TS, tumor suppressor of tumor associated genes; TAG_OTHER, other genes of tumor associated genes.

data collected by Schriml *et al* (26), membrane metallo-endopeptidase (MME), MMP-2 and MMP-9 were identified as DEGs associated with proliferative diabetic retinopathy.

PPI network construction and sub-network detection. A PPI network of DEGs was constructed based on the STRING database. The top 6 genes with degree >5 were epidermal growth factor receptor (EGFR), proto-oncogene tyrosine protein kinase Fyn (FYN), c-JUN (JUN), glucagon (GCG), c-Myc (MYC) and CD44 (Fig. 1). A sub-network involving 70 gene nodes was identified with EGFR (degree=12) as the central gene (Fig. 2). Genes in this sub-network primarily participate in various types of cancer and cancer-associated processes, including signaling pathways [such as the epidermal growth factor receptor (ErbB) and gonadotropin-releasing hormone (GnRH) signaling pathways], and immune response pathways (Table IV).

Discussion

The SPN is a grossly solid or solid and cystic malignant epithelial neoplasm, where poorly cohesive cells surrounding

delicate blood vessels form degenerative pseudopapillae (27). The present study aimed to investigate the potential mechanisms of SPN, and identify genes to use as diagnostic markers and understand tumor phenotype and behavior, aiding in the development of molecularly-targeted therapy. A total of 1,420 DEGs were identified between SPN and control samples. Following PPI network analysis, EGFR, FYN, JUN, GCG, MYC and CD44 were identified. GO functional enrichment analysis and KEGG pathway enrichment analysis indicated that these were predominantly enriched in the ErbB, GnRH and Wnt signaling pathways.

MME, MMP-2 and MMP-9 were upregulated and identified to be associated with proliferative diabetic retinopathy. MME, also termed CD10, encodes MME, which is a 100-kD type II transmembrane glycoprotein. CD10 is associated with various types of cancer, including gastric (28), breast (29), colorectal (30) and pancreatic cancer (31). Ikenaga *et al* (31) demonstrated that CD10⁺ pancreatic stellate cells promote the invasion of pancreatic cancer cells and secrete MMP-3, contributing to the progression of pancreatic cancer. Therefore, CD10 may be an optimal therapeutic target in the treatment of SPN. MMP-2 and MMP-9 both encode members of the MMP

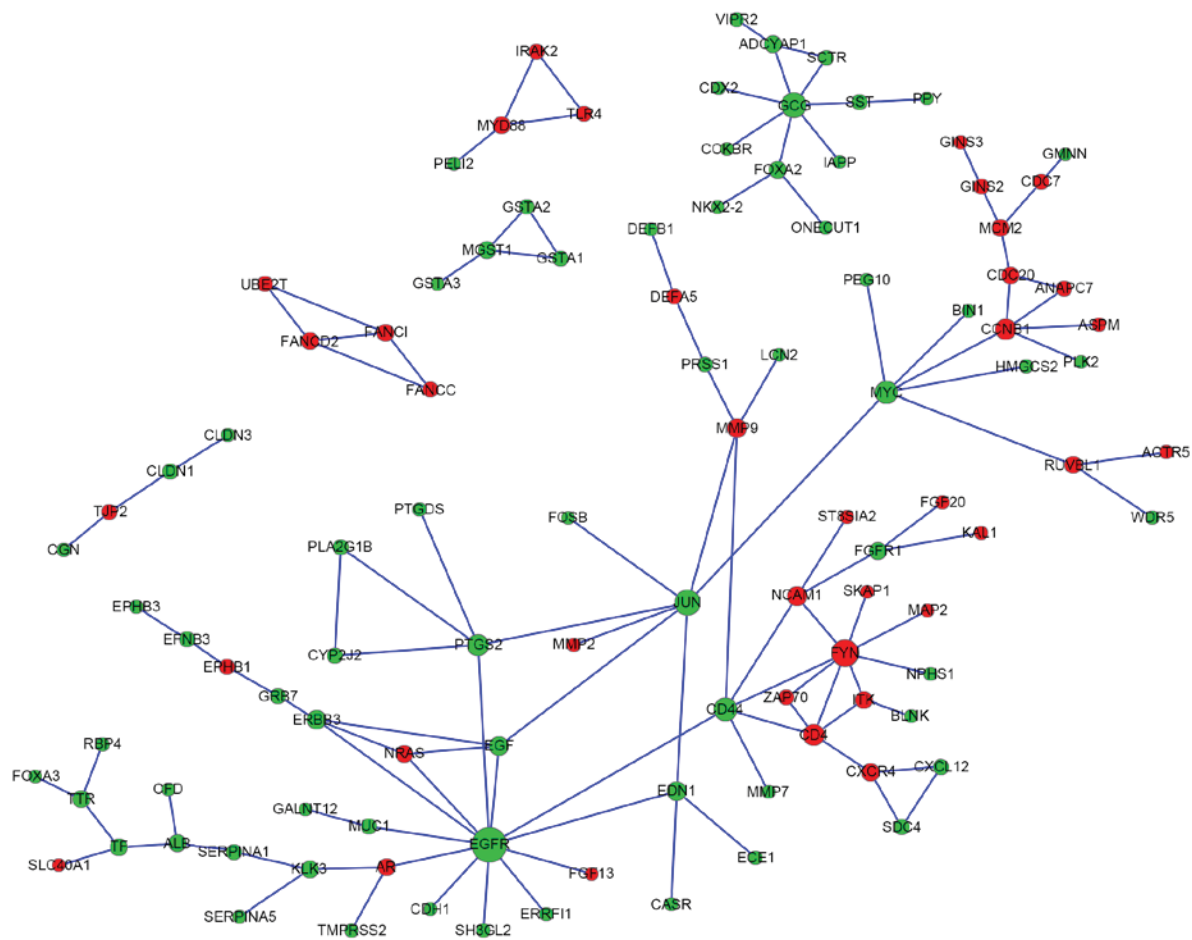


Figure 1. Protein-protein interaction network of DEGs between solid pseudopapillary neoplasm of pancreas and control samples. Red nodes represent upregulated DEGs; green nodes represent downregulated DEGs. DEG, differentially expressed gene.

family, a major family of proteases involved in remodeling the extracellular matrix. Activation of MMP-2 and MMP-9 has been demonstrated to be associated with the metastasis process and local recurrence rate (32). Inhibiting MMP activation blocks the metastasis process and is an effective therapeutic approach (33). El-Ghlban *et al* (34) demonstrated that the fusion form of chlorotoxin (CTX), which is formed by CTX and the human IgG-Fc domain, may be an effective treatment for pancreatic cancer, as it binds to MMP-2 and suppresses its expression. Thus, MMP-2 also has the potential to be used as therapeutic target in the treatment of SPN.

PPI network analysis demonstrated that the expression levels of EGFR, FYN, JUN, GCG, MYC and CD44 were significantly increased in SPN samples compared with controls, indicating that these genes are associated with SPN. EGFR encodes the transmembrane glycoprotein epidermal growth factor, a member of the protein kinase superfamily (35). It induces receptor dimerization and tyrosine autophosphorylation, and is overexpressed in pancreatic cancer (36,37). Phosphorylation of EGFR initiates modules including the mitogen-activated protein kinase (MAPK) pathway, the phosphatidylinositol 3-kinase/Akt pathway and MAPK/extracellular signal-related kinase (ERK) pathway, all of which have been proven to affect cell survival, metastasis, proliferation, invasion and induction of cancer (38). JUN encodes c-Jun, a proto-oncogene and basic region-leucine zipper transcription

factor involved in multiple cellular processes through the formation of various dimeric complexes (39). The direct combination of JUN transcriptional activation and cyclin D1 provides a molecular link between growth factor signaling and the changes in cell cycle proteins that drive the G₁/S transition. Previous studies have demonstrated that cyclin D1 activates the MAPK/ERK pathway and induces cancer (40,41). MYC encodes c-Myc, an avian myelocytomatosis viral oncogene homolog that participates in apoptosis, adhesion, differentiation, growth and migration (42). Overexpression of MYC in pancreatic cancer (43,44) has been demonstrated previously. MYC activation results in upregulation of G₁-specific cyclins and cyclin-dependent kinases, and inhibits negative regulatory factors of cell cycle progression. Cells were therefore able to pass through the restriction point and progress from the G₁ to the S phase (45).

It has been previously demonstrated that the Wnt signaling pathway is involved in the tumorigenesis of SPN (7), and KEGG pathway analysis of all the upregulated DEGs indicated enrichment of the Wnt signaling pathway. The ErbB and GnRH signaling pathway were also demonstrated to be significantly enriched. The ErbB protein family contains four structurally-associated receptor tyrosine kinases including ErbB-1/HER1/EGFR, ErbB-2/HER2, ErbB-3/HER3 and ErbB-4/HER4. Excessive ErbB signaling is associated with the development of various types of solid tumor (46). Previous

Table IV. Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis of differentially expressed genes in the identified sub-network.

KEGG pathway	Gene counts	P-value	Gene
Bladder cancer	7	2.11E-08	<i>MYC, EGFR, EGF, CDH1, MMP9, NRAS, MMP2</i>
Endometrial cancer	5	4.19E-05	<i>MYC, EGFR, EGF, CDH1, NRAS</i>
Melanoma	5	0.0001884	<i>EGFR, EGF, CDH1, NRAS, FGF13</i>
Cell cycle	6	0.0003266	<i>CDC20, CCNB1, CDC7, MYC, ANAPC7, MCM2</i>
Prostate cancer	5	0.0005422	<i>AR, KLK3, EGFR, EGF, NRAS</i>
ErbB signaling pathway	6	4.57E-05	<i>JUN, MYC, EGFR, EGF, ERBB3, NRAS</i>
GnRH signaling pathway	5	0.0009664	<i>JUN, EGFR, PLA2G1B, NRAS, MMP2</i>
T cell receptor signaling pathway	6	0.0001534	<i>JUN, CD4, FYN, ZAP70, NRAS, ITK</i>
Axon guidance	7	4.76E-05	<i>CXCR4, FYN, EFNB3, CXCL12, EPHB1, NRAS, EPHB3</i>
Pathways in cancer	12	3.841E-06	<i>AR, JUN, KLK3, MYC, EGFR, EGF, CDH1, MMP9, PTGS2, NRAS, MMP2, FGF13</i>

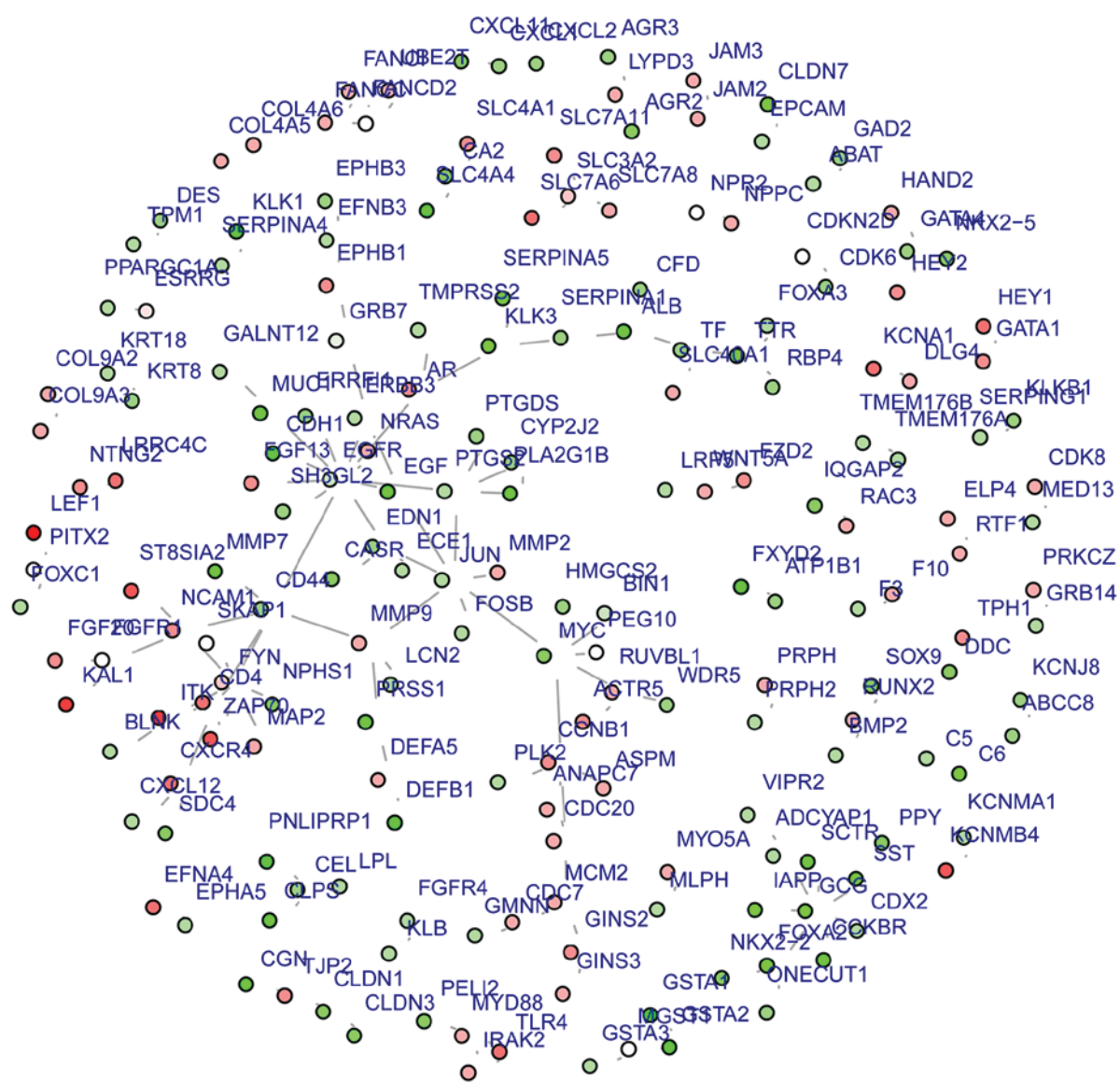


Figure 2. Significant sub-networks of the protein-protein interaction network. The depth of color is proportional to log₂ fold-change of DEGs. Red nodes represent upregulated DEGs and green nodes represent downregulated DEGs. Square nodes represent genes with low importance and circular nodes represent genes with high importance. DEG, differentially expressed gene.

clinical studies have demonstrated that ErbB-1 and ErbB-2 expression is altered in numerous types of human cancer, and the resultant excessive signaling may be critical factors in tumor etiology and progression (47). It has been previously demonstrated that ErbB-1 induces cancer (48), and ErbB-2 homodimers alone may contribute to malignancy (49). However, a number of observations suggest that ErbB-2 may potentiate ErbB-1 signaling (47).

GnRH encodes a pre-prohormone, consisting of a 23-amino-acid signal peptide. The GnRH receptor (GnRH-R) is currently treated as a molecular target in the treatment of hormone-dependent tumors. GnRH-R activation, coupled to $G_{q/11}$ -G $\beta\gamma$ proteins, leads to elevation of intracellular Ca^{2+} levels, altered cytoskeletal function and changes in protein kinase activity, including protein kinase C, mitogen activated serine/threonine kinases and stress-activated kinases (50). Sikora and Vali (51) previously demonstrated that, in addition to the Wnt- β -catenin pathway, additional pathways intervening with growth factor signaling, key kinases and inherent converging points in the signaling machinery also affect SPN.

To conclude, in order to illustrate the pathological mechanisms of SPN, gene expression profiles of 19 samples were downloaded and analyzed. Gene functional annotation analysis demonstrated that the genes MME, MMP-2 and MMP-9, which are involved in proliferative diabetic retinopathy, are also involved in SPN. Through PPI network and module analysis, the genes EGFR, FYN, JUN, GCG, MYC and CD44 were identified as potential key SPN genes. In addition, the ErbB and GnRH signaling pathways may be involved with SPN progression. Furthermore, the above DEGS might function as potential targets for the further gene treatment of SPN.

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