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Identification of Key Genes and Pathways in Colorectal Cancer Gene Expression Profile by Bioinformatics

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Abstract

The aim of this study was to identify potential key candidate genes and uncover their potential mechanisms in colorectal cancer. The gene expression profiles of GSE8671, GSE32323 and GSE21510 were downloaded from the GEO database, including 68 colorectal cancer and 74 normal samples. The differentially expressed genes (DEGs) between the two types of samples were identified by R language. The gene ontology functional and pathway enrichment analyses of DEGs were performed using the DAVID software followed by the construction of PPI network. Hub gene identification and pathway enrichment analyses of the modules were performed. The DEGs were mainly involved in cell division and cell proliferation. The top hub genes such as IL8, MYC were identified from the PPI network. Sub-networks revealed hub genes were involved in significant pathways, including chemokine signaling pathway and cell cycle. These hub genes may be used as potential targets for colorectal cancer diagnosis and treatment.

Keywords

Colorectal Cancer, Bioinformatics Analysis, Network Module, Enrichment Analysis

1. Introduction

Colorectal cancer (CRC) is the third most common form of cancer in developed countries and, despite the improvements achieved in its treatment options, remains as one of the main causes of cancer-related death [1]. Most colorectal cancers are due to old age and lifestyle factors with only a small number of cases due to underlying genetic disorders. Statistically significant interactions between the nutritional factors, family history of colorectal cancer and colorectal cancer risk are reported [2]. In the last two decades, great efforts have been made in the treatment of CRC due to the approval of new target agents for cytotoxic drugs. Unfortunately, a large percentage of patients present with metastasis at the time of diagnosis or relapse after a few months. The complex molecular heterogeneity of this disease is not completely understood; to date, there is a lack of predictive biomarkers that can be used to select subsets of patients who may respond to target drugs [3]. Many researchers are actively pursuing molecular biological analyses of the mechanisms involved in the onset and progression of CRC. It is well know that genetic and epigenetic abnormalities lead to characteristic gene expression profiles that are strongly linked to clinical outcomes. Several pathways underlie CRC pathogenesis, however, the main 3 routes are: the chromosomal instability pathway (CIN), the microsatellite instability pathway (MSI), and the serrated pathway. The majority of CRCs arise from the CIN pathway, which is characterized by defects in chromosomal segregation, telomere stability, and the DNA damage response. On the other hand, MSI derives from the loss of DNA mismatch repair and is found in about 15% of all CRCs [4]. Therefore, it is important that the molecular mechanism of CRC is illuminated for the development of more effective diagnostic and therapeutic strategies.

Microarrays are one of the high-throughput platforms for analysis of gene expression and served as key tools in medical

oncology with great clinical applications, such as molecular diagnosis and classification of cancers. In the last decade, a large number of gene expression profiling researches on CRC have been reported by microarray technology and revealed many differentially expressed genes (DEGs) involved in different pathways, biological processes, or molecular functions [5, 6]. However, comparative analysis of the DEGs in independent studies shows a relatively limited degree of overlap, and no reliable biomarker profile discriminating cancerous from normal tissue has been identified [7]. However, the integrated bioinformatics methods combining with expression profiling techniques will be innovative and might solve the disadvantages.

This study used microarray gene expression profile to identify biomarkers and pathways involved in CRC. On the basis of analyzing their biological functions and pathways, we may show the further insight of CRC development at molecular level and explored the potential candidate biomarkers for diagnosis, prognosis, and drug targets.

2. Materials and Methods

2.1. Microarray Data

The gene expression profiles of GSE8671, GSE32323 and GSE21510 were downloaded from GEO database. The dataset of GSE8671 was based on GPL570 platform (Affymetrix Human Genome U133 Plus 2.0 Array) and included 32 CRC tissues and 32 normal tissues. The dataset of GSE32323 was based on GPL570 platform (Affymetrix Human Genome U133 Plus 2.0 Array) and included 17 CRC tissues and 17 normal tissues. The dataset of GSE21510 was based on GPL570 platform (Affymetrix Human Genome U133 Plus 2.0 Array) and included 19 homogenized CRC tissues and 25 homogenized normal tissues. These 3 datasets were chosen for integrated analysis in this study including 68 CRC samples and 74 normal samples.

2.2. Data Preprocessing and DEGs Screening

The raw data was preprocessed by Affy package (R/Bioconductor) of R language following the three steps: background adjustment, quantile normalization, logarithmic transformation and finally summarization. Then the expression matrix with probe level was transformed to matrix with gene level based on annotation files. Multiple Linear Regression limma was applied for DEGs analysis [8]. The ComBat function of sva package was used to remove known batch effects from microarray data [9]. Volcano plot was used to display both average fold change and P-value, which was generated by using ggplots package of R language. DEGs were identified with classical t test, statistically significant DEGs were defined with $P < 0.01$ and log2-fold change ($\log_2FC$) > 1 as the cut-off criterion.

2.3. Hierarchical Clustering Analysis

After extracting the expression values from the gene expression profile, a bidirectional hierarchical clustering heatmap was constructed using gplots package of R language.

2.4. Functional and Pathway Enrichment Analysis

The database for Annotation, Visualization and Integrated Discovery (DAVID) was used to classify significant DEGs by their biological processes, molecular functions, or cellular components using Gene Ontology consortium reference (GO) and the significant transcripts (Benjamini-Hochberg FDR <0.05) were identified using the Functional Annotation clustering tool. The DAVID database was also used to perform pathway enrichment analysis with reference from Kyoto Encyclopedia of Genes and Genomes (KEGG) database website and Benjamini-Hochberg FDR <0.05 as a cut-off point.

2.5. PPI Network Construction and Module Analysis

In the construction of the PPI networks, the Search Tool for the Retrieval of Interacting Genes (STRING) version 10.5 (http://www.string-db.org/) was used. This is a web biological database for prediction of known and unknown protein interaction relationships. The DEGs with required confidence (combined score) >0.4 were selected, and then the PPI network was constructed and visualized using Cytoscape software version 3.5.0. The plug-in Molecular Complex Detection (MCODE) was used to screen the modules of PPI network in Cytoscape [10]. The criteria were set as follows: MCODE scores>4 and number of nodes>4. Moreover, the function and pathway enrichment analysis were performed for DEGs in the modules. $P<0.05$ was considered to have significant differences.

3. Results

3.1. Identification of DEGs

Using $P < 0.01$ and $|logFC| > 1$ as cut-off criterion, a total of 2274 DEGs were identified from the three profile datasets including 1159 up-regulated genes and 1115 down-regulated genes in the colorectal cancer tissues compared to normal colon tissues, which was showed by volcano plot (Figure 1). The gene expression values were extracted and a hierarchical clustering heat map was plotted to present the DEGs (Figure 2).

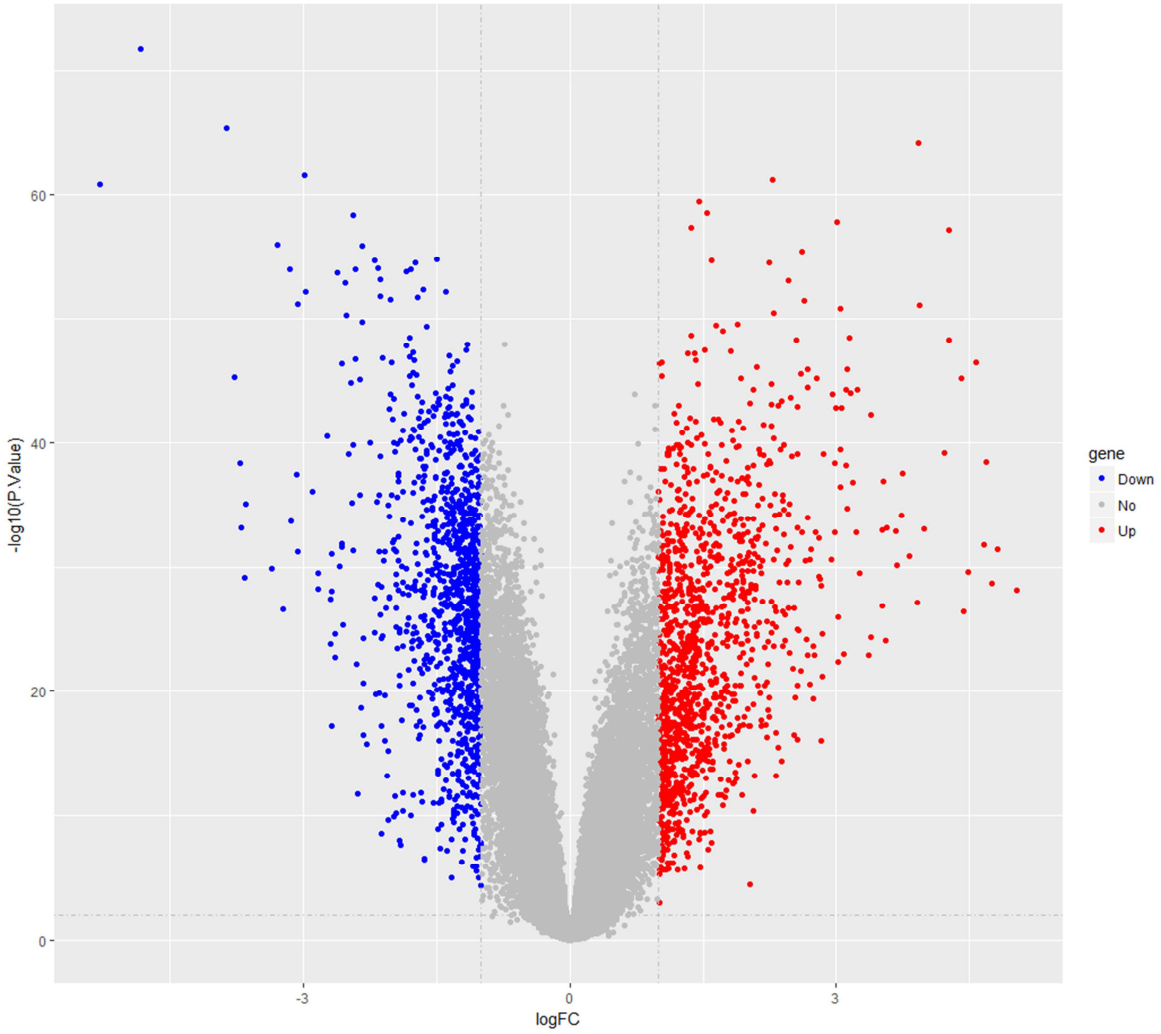

Figure 1. *Volcano plot showing all the genes expression change in normal or colorectal cancer tissues. Grey represents no change in expression, blue represents down- regulation, and red represents up-regulation.*

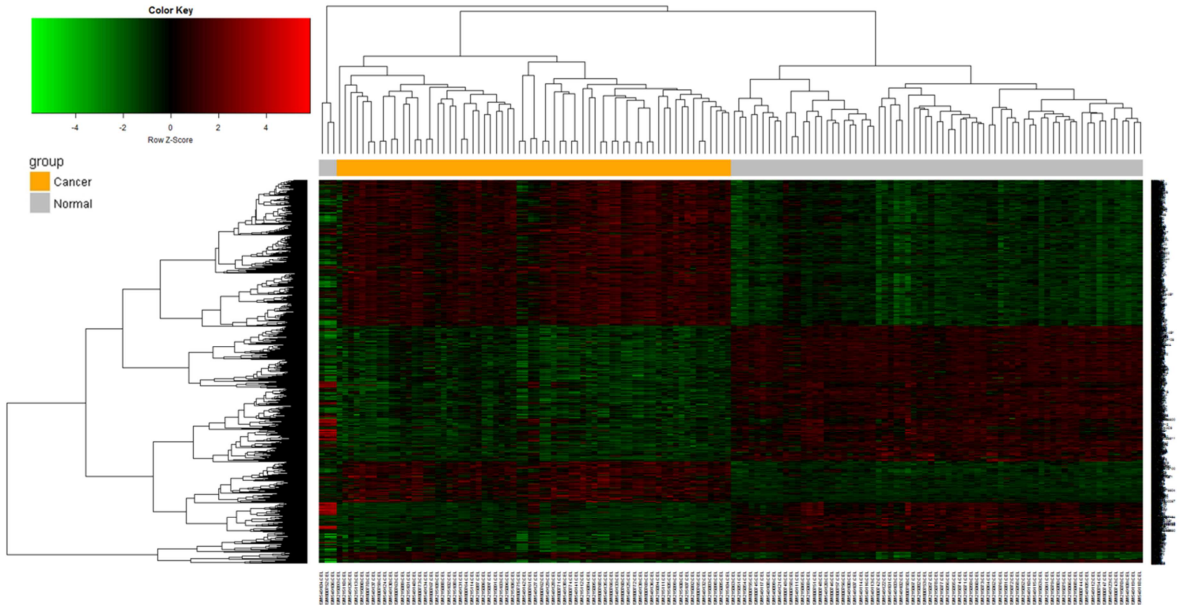

Figure 2. *Heat map showing up-regulated and down-regulated differentially expressed genes (DEGs) in normal or colorectal cancer tissues. The expression values are log_2 fold changes (>1 or <−1) between normal tissues and colorectal cancer tissues. Green represents down-regulation and red represents up-regulation.*

3.2. GO Functional and Pathway Enrichment Analysis

GO analysis results showed that the DEGs were significantly enriched in cell component, including nucleoplasm, extracellular space, extracellular exosome, and so on. For biological processes, the DEGs were significantly enriched in cell division, DNA replication, G1/S transition of mitotic cell cycle, mitotic nuclear division and cell proliferation. In addition, GO analysis also displayed that the DEGs were significantly enriched in protein binding and chemokine activity for molecular function (Table 1).

KEGG signaling pathway analysis results showed that the DEGs were significantly enriched in cell cycle, DNA replication, mineral absorption, ribosome biogenesis in eukaryotes, p53 signaling pathway, pathways in cancer (Table 1).

Table 1. *GO and pathway enrichment analysis of the DEGs (top 5 in each category).*

Category	ID	Term	Count	P-value	FDR
BP	GO:0051301	cell division	79	2.71E-14	5.14E-11
BP	GO:0006260	DNA replication	47	1.37E-13	2.60E-10
BP	GO:0000082	G1/S transition of mitotic cell cycle	37	1.90E-13	3.60E-10
BP	GO:0007067	mitotic nuclear division	62	1.94E-13	3.68E-10
BP	GO:0008283	cell proliferation	73	1.37E-10	2.61E-07
CC	GO:0005654	nucleoplasm	352	2.11E-14	3.19E-11
CC	GO:0005615	extracellular space	189	1.90E-11	2.88E-08
CC	GO:0070062	extracellular exosome	338	5.83E-11	8.82E-08
CC	GO:0005829	cytosol	385	1.89E-10	2.86E-07
CC	GO:0000776	kinetochore	25	5.23E-08	7.90E-05
MF	GO:0005515	protein binding	912	3.34E-14	5.55E-11
MF	GO:0008009	chemokine activity	17	2.43E-06	0.004
MF	GO:0008201	heparin binding	33	8.32E-06	0.014
MF	GO:0003688	DNA replication origin binding	8	9.70E-06	0.016
KEGG pathway	hsa04110	Cell cycle	43	1.05E-13	1.39E-10
KEGG pathway	hsa03030	DNA replication	16	4.66E-07	6.17E-04
KEGG pathway	hsa04978	Mineral absorption	16	1.69E-05	0.022
KEGG pathway	hsa03008	Ribosome biogenesis in eukaryotes	23	2.05E-05	0.027
KEGG pathway	hsa04115	p53 signaling pathway	19	4.85E-05	0.043

Note: GO, gene ontology; DEGs, differentially‐expressed genes; KEGG, Kyoto Encyclopedia of Genes and Genomes; BP, biological process; CC, cell component; MF, molecular function; Count, numbers of DEGs enriched in each term; FDR, false discovery rate (Q-value).

3.3. PPI Network Construction and Hub Gene Identification

Based on the information in the STRING database, PPI relationships were obtained and the hub genes or proteins in the networks with connectivity degree > 10 were identified (Figure 3-A). The top 18 hub nodes with higher degrees were screened (Table 2). These hub genes included interleukin 8 (IL8), v-myc avian myelocytomatosis viral oncogene homolog (MYC), somatostatin (SST), chemokine (C-X-C motif) ligand 12 (CXCL12), cyclin-dependent kinase 1 (CDK1), and so on (Table 2). Among these genes, IL8 showed the highest node degree, which was 24.

Table 2. *The hub proteins in the protein–protein interaction network.*

Gene symbol	Gene title	Degree
IL8	interleukin 8	24
MYC	v-myc avian myelocytomatosis viral oncogene homolog	21
SST	somatostatin	19
CXCL12	chemokine (C-X-C motif) ligand 12	16
CD44	CD44 molecule (Indian blood group)	15
PHLPP2	PH domain and leucine rich repeat protein phosphatase 2	15
CDK1	cyclin-dependent kinase 1	14
CXCL1	chemokine (C-X-C motif) ligand 1	14
PYY	peptide YY	14
LPAR1	lysophosphatidic acid receptor 1	13
CXCL5	chemokine (C-X-C motif) ligand 5	12
GCG	glucagon receptor	12
TIMP1	TIMP metallopeptidase inhibitor 1	11
CXCL2	chemokine (C-X-C motif) ligand 2	11
CXCL11	chemokine (C-X-C motif) ligand 11	11
CXCL3	chemokine (C-X-C motif) ligand 3	11
CXCL13	chemokine (C-X-C motif) ligand 13	11
INSL5	insulin-like 5	11

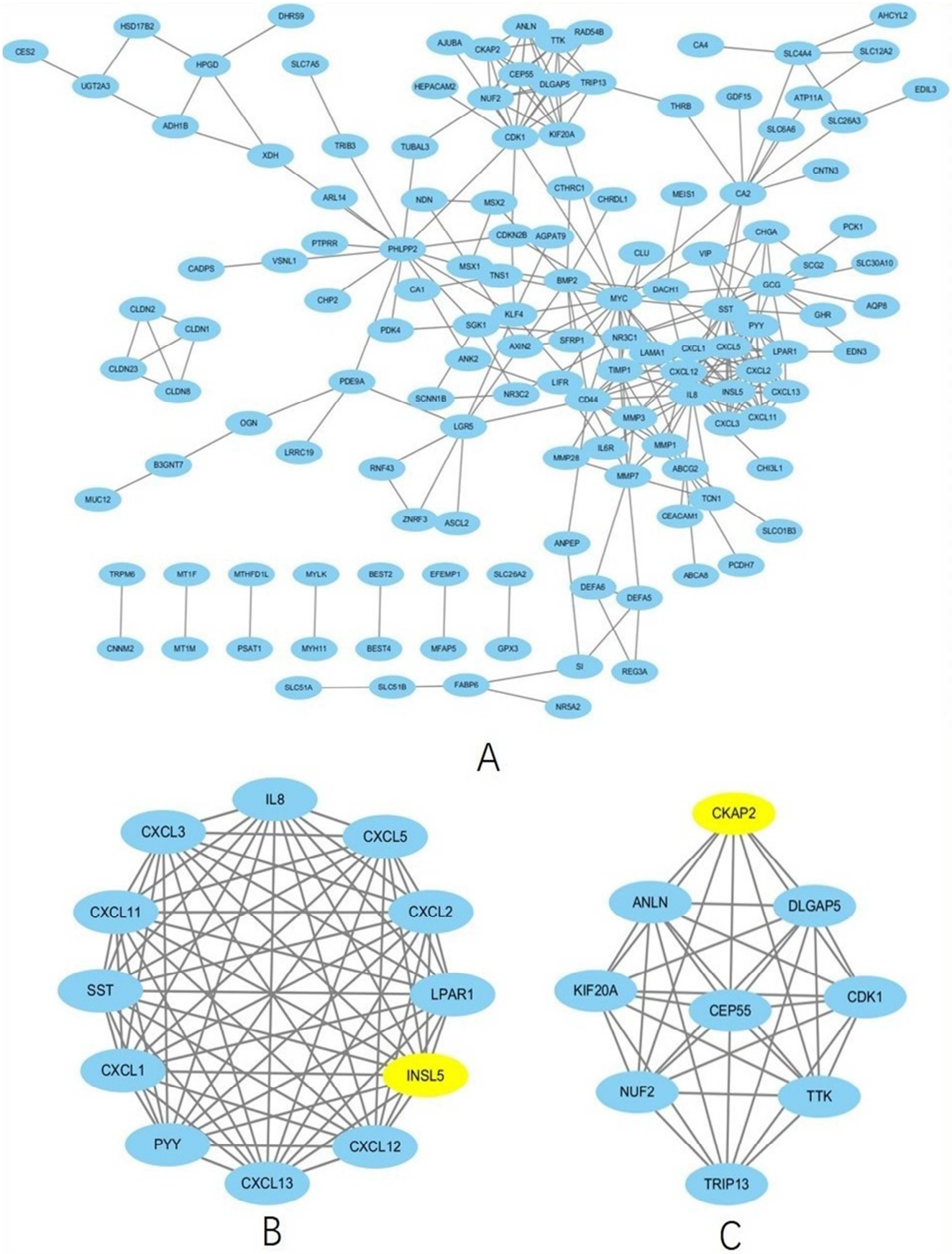

Figure 3. *Differentially expressed genes (DEGs) protein–protein interaction (PPI) network complex and modular analysis. (A) PPI network was constructed and visualized using Cytoscape software. (B) Module-1, yellow standing for the seed node in the module by module analysis. (C) Module-2, yellow standing for the seed node in the module by module analysis.*

3.4. Module Analysis

Three modules with MCODE scores>4 and nodes>4 were selected in the PPI networks. Cluster-1 (MCODE scores=12) was with 12 nodes and 66 edges, and the seed node was INSL5. Cluster-2 (MCODE scores=8.75) was with 9 nodes and 35 edges, and the seed node was CKAP2. Cluster-3 (MCODE scores=5) was with 5 nodes and 10 edges, and the seed node was MMP7 (Figure 3-B, Figure 3-C). The seed node was INSL5, CKAP2, MMP7 in the Cluster-1, Cluster-2 and Cluster-3, respectively. In addition, the KEGG pathway analysis revealed that the hub genes in modules were significantly enriched in pathways including chemokine signaling pathway, TNF signaling pathway, legionellosis, cell cycle, Epstein-Barr virus infection and cytokine-cytokine receptor interaction (Table 3).

Table 3. *The KEGG pathway enrichment analysis in the top 2 modules genes.*

Category	Term	Count	P-value	Genes
	hsa04062: Chemokine signaling pathway	7	9.40E-09	CXCL1, CXCL5, CXCL13, CXCL3, CXCL2, CXCL11, CXCL12
	hsa05134: Legionellosis	3	0.0016	CXCL1, CXCL3, CXCL2
Cluster-1	hsa05132: Salmonella infection	3	0.0038	CXCL1, CXCL3, CXCL2
	hsa04668: TNF signaling pathway	3	0.0061	CXCL1, CXCL3, CXCL2
	hsa04060: Cytokine-cytokine receptor interaction	3	0.0271	CXCL13, CXCL11, CXCL12
Cluster-2	hsa04110: Cell cycle	2	0.0179	CDK1, TTK

Note: KEGG: Kyoto Encyclopedia of Genes and Genomes, P<0.05.

4. Discussion

Many studies have been performed to disclose the causes and underlying mechanisms of colorectal cancer formation and progression during the past decades, but the incidence and mortality of CRC is still very high in the world, because most studies focus on a single genetic event or the results are generated from a single cohort study [11]. Our study integrated three cohorts profile datasets from different groups, utilized bioinformatics methods to deeply analyze these datasets, and identified 2274 commonly changed DEGs. The number of up-regulated genes was equal to the down-regulated genes. In the hierarchical clustering analysis, we found three normal samples (GSM549104, GSM800752, and GSM549106) were not classified as the normal group. Therefore, the three normal samples were removed from all the microarray data to control the chip quality in the next analysis.

There is an increasing interest in searching for networks of genes, instead of single genes, contributing to the etiology of complex diseases, since changes in biological characteristics require coordinate variation in expression of gene sets. Enrichment analysis tools, which estimate overrepresentation of particular gene categories or pathways in a gene list, are a useful approach in this direction [7]. In order to better understand the interactions of DEGs, we further carried out GO and KEGG pathway analysis. It was showed that DEGs were mainly involved in cell division, DNA replication, G1/S transition of mitotic cell cycle, mitotic nuclear division and cell proliferation by the GO term analysis. Development and progression of colorectal cancer is closely related to the abnormal function of cell cycle and cell proliferation regulators [12]. Moreover, the enriched KEGG pathways of DEGs included cell cycle, DNA replication, mineral absorption, and p53 signaling pathway. Nutrients can directly affect fundamental cellular processes and are considered among the most important risk factors in CRC [13]. Additionally, the p53 signaling pathway has been highly focused on CRC [14].

IL-8 and MYC were selected with the high connective degree after analyzing the hub genes from the DEGs PPI network. CD44, CXCL12 and CDK1 were also identified as hub genes, this is consistent with the previous study [15]. IL-8, also known as neutrophil chemotactic factor, has two primary functions. It induces chemotaxis in target cells, primarily neutrophils but also other granulocytes, causing them to migrate toward the site of infection. IL-8 also induces phagocytosis once they have arrived. IL-8 is also known to be a potent promoter of angiogenesis. In recent years inflammation has been considered on the mechanisms involved in the early stages of colorectal carcinogenesis [16]. Indeed, tissue injury resulting from infectious, mechanical, or chemical agents may elicit a chronic immune response resulting in cellular proliferation and regeneration. If the immune response fails to resolve injury, a microenvironment rich in cytokines, growth factors, and products of cellular respiration sustains a prolonged proliferation in attempt to repair, resulting in the accumulation of genetic errors and continued inappropriate proliferation [17]. Evidence supports a role for inflammatory responses in the development of colorectal cancer [18-21]. Furthermore, it is generally accepted today that tumor growth is angiogenesis-dependent and that every increment of tumor growth requires an increment of vascular growth [22]. MYC is a regulator gene that codes for a transcription factor. The protein encoded by this gene is a multifunctional, nuclear phosphoprotein that plays a role in cell cycle progression, apoptosis and cellular transformation. MYC is a very strong proto-oncogene and it is very often found to be up-regulated in many types of cancers. Multiomics-based analyses of paired normal and tumor tissues from 275 patients with colorectal cancer revealed that metabolic alterations occur at the adenoma stage of carcinogenesis, in a manner not associated with specific gene

mutations involved in colorectal carcinogenesis [23]. MYC expression induced at least 215 metabolic reactions by changing the expression levels of 121 metabolic genes and 39 transporter genes. Further, MYC negatively regulated the expression of genes involved in mitochondrial biogenesis and maintenance but positively regulated genes involved in DNA and histone methylation [23]. On the basis of The Cancer Genome Atlas (TCGA), MYC, WT1, mir-34a, and LEF1 were located in the central hub of the network of TF-gene-miRNA in colon cancer by bioinformatics analysis [24].

Module analysis of the PPI network revealed that the development of CRC was associated with chemokine signaling pathway, TNF signaling pathway, cell cycle, and so on. The first module or cluster consisting of 12 genes, including IL8, SST, C-X-C Motif Chemokine Ligend (CXCL) family members (CXCL12, CXCL1, CXCL5, CXCL2), ISNL5, were listed at the top of the most changed genes, and their biological functions are mainly involved in cell inflammation response. It was interesting that ISNL5 is identified as the seed node in the cluster-1. Insulin-like peptide 5 (INSL5) is a member of the insulin superfamily, and is a potent agonist for RXFP4. INSL5 is expressed in enteroendocrine cells (EECs) along the colorectum with a gradient increase toward the rectum. RXFP4 is ubiquitously expressed along the digestive tract [25]. Exogenous INSL5 did not affect the proliferation of human colon cancer cell lines, and chemically-induced colitis in INSL5 null mice did not show any significant changes in inflammation or mucosal healing compared to wild-type mice. In contrast, all of the rectal neuroendocrine tumors examined co-expressed INSL5 and RXFP4. INSL5 may be a unique marker of colorectal EECs, and INSL5-RXFP4 signaling might play a role in an autocrine/paracrine fashion in the colorectal epithelium and rectal neuroendocrine tumors [25]. As another member of the insulin superfamily, the insulin-like peptide 3 (INSL3) can promote early tumor cell invasiveness in human thyroid carcinoma cells by enhancing their metabolic activity and elastin-degrading potential [26].

The second module consisting of 9 genes, including CDK1, TTK, and CKAP2, were listed at the top of the most changed genes, and their biological functions are mainly involved in cell cycle. Cytoskeleton-associated protein 2 (CKAP2) is identified as the seed node in the cluster-2. CKAP2 is known to be highly expressed in primary human cancers as well as most cancer cell lines. CKAP2 functions as microtubule stabilizer and probably as cell proliferation inducer, indicating that CKAP2 might be a potential anticancer target [27]. Previous study has indicated that CKAP2 RNA is a promising target for anticancer approach based on trans-splicing ribozyme-mediated RNA replacement [28]. Overexpression of CKAP2 enhanced proliferation and mobility of carcinoma cells through the FAK-ERK2 signaling pathway [29]. Proliferation activity has already been established as a prognostic marker or as a marker for anticancer drug sensitivity. CKAP2 staining has recently been introduced as a marker of proliferation activity in gastric cancer and breast cancer tissues by immunohistochemistry analysis [30].

5. Conclusions

The study has identified 2274 DEGs candidate genes using multiple cohorts profile datasets and integrated bioinformatical analysis, and filtered 180 gene nodes in DEGs protein–protein interaction network complex, and finally found 18 mostly changed hub genes, which significant enriched in several pathways, mainly associated with chemokine signaling pathway, TNF signaling pathway, cell cycle in colorectal cancer. These findings could significantly improve understanding of the cause and underlying molecular events in CRC, these candidate genes and pathways could be therapeutic targets for CRC.

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