Original Article
Screening feature genes of osteosarcoma with DNA microarray: a bioinformatic analysis

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Abstract: To screen differentially expressed genes (DEGs) of Osteosarcoma (OS) by using the microarray expression profiles of tissues of normal person and patients with OS for early diagnosis and effective treatment of OS. We downloaded the gene expression profile of GES16088 from Gene Expression Omnibus database, including twenty samples from fourteen patients diagnosed with osteosarcoma and six normal samples as control groups. We identified the DEGs by Affy package in R language. Bioinformatic methods were used for further analysis of the screened DEGs. Firstly, cluster analysis was performed on the selected DEGs for comparison of the expression degree. After protein-protein interaction (PPI) network of differentially expressed genes was constructed by STRING, we analyzed gene functions with DAVID and WebGestalt. Compared with the control, we screened three distinctly up-regulated genes. These DEGs had close relationship with transmission signaling pathway, organ and system development. The up-regulated gene COL was the most representative genes among the DEGs. The screened DEGs have a great significance on studying mechanism of osteosarcoma. It might distinguish normal and pathological tissues of OS and thus become target genes for monitoring, diagnosis and treatment of the OS. It has the potential to use in clinic.

Keywords: Osteosarcoma, differentially expressed genes, protein-protein interaction, gene ontology enrichment

Introduction

Osteosarcoma (OS) is the most common malignant bone tumor. It occurs most frequently in adolescents among individuals aged >60 years [1]. Eighty percent of this tumor will metastasize to the lungs or liver, and as a result, patients generally need chemotherapy for survival possibility. In the last two decades there have been no concrete developments in their systemic treatment. Most osteosarcoma epidemiology studies have been embedded in large analysis of all bone tumors or focused on cases occurring in adolescence [2]. Despite the considerable improvement in OS in the last few years, given the fact that almost eighty percent of the patients with OS can hold their morbid limbs and survival rate of OS rise from twenty percent to eighty percent, more than half patients still die from the metastases and relapse of the disease [3]. Recently, with the development of molecular biology of tumor, therapeutic strategies on genes have been reported in extensive studies, which has revealed good potential in clinical appliance [4-6]. To identify OS-related specific genes for early diagnosis of OS, we attempt to find out significantly differently expressed genes in OS by analyzing serum samples from patients with OS and normal tissue samples form control groups, in the hope to select out potential biomarkers for early diagnosis of OS.

Early study showed that interleukin-1 beta (IL-1B) is crucially involved in osteosarcoma carcinogenes and revealed the association between IL-1B gene polymorphisms and osteosarcoma risk [7]. In Song GH's investigation [8], SCUBE3 is reported to up-regulated in primary osteosarcoma tissues to promote the proliferation of osteosarcoma cells, illuminating that high SCUBE3 expression in osteosarcoma tissues could be associated with a poor prognosis of the patients, further suggesting that SCUBE3 can serve as a potential therapeutic target for osteosarcoma.

Despite all the considerate study, detailed descriptions of osteosarcoma incidence and biomarkers of patients of all ages and ethnicities are not available. In this study, we are in the hope for finding genes specially expressed in OS by analyzing expression profiling from normal persons and specimen with OS. The screened DEGs may have the potential to become candidate target genes of the OS, and may provide evidence for early diagnosis and treatment of OS.

DNA microarray has been used to investigate the pathogenesis in the breast cancer and pancreatic cancer [9, 10]. In this study, we downloaded the gene expression profile of OS tissues and patient-matched normal tissues. The differentially expressed genes (DEGs) in OS tissue were selected by using the expression profiling of normal tissue and OS tissue. In addition, we then used bioinformatic methods to analyze the screened DEGs. We anticipate that our work could improve the understanding of the underlying molecular mechanisms of OS and could provide novel insights for the early diagnosis and medication control of OS.

Materials and methods

Data profiling of OS

We extracted the gene expression profile of GES16088 [11] from GEO (Gene Expression Omnibus) database, including twenty samples from fourteen patients diagnosed with osteosarcoma and six normal samples as control groups. The data platform was at GPL96 [HG-U133A] Affymetrix Human Genome U133A Array.

Data preprocessing and differential expression analysis

Affy package in R language was used to transform original data into the form of expression data [12]. Missing data was imputed [13] and finally all expression profile was normalized by the method of median normalization [14].

Three statistic testing methods of Multtest package in R language [15], namely, T testing, Wilcox testing and Fisher testing were strictly used to identify DEGs in expression values from patients and normal sample tissues. Since multi-test might induce too much false positive results, we used the method of BH [16], which can adjust the raw *P*-values into false discovery rate (FDR) to circumvent the problem. The FDR<0.05 and |logFC|>1 were used as the cut-off criteria.

STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) [17, 18] online was used to construct PPI network among DEGs. Genes with high degree of coefficient were selected to construct protein-protein interaction network by Cytoscape [19]. Igraph package in R language was used to calculate the connectivity degree of nodes.

Protein-protein interaction (PPI) network construction

The protein-protein interactions (PPIs) research could reveal the functions of proteins at the molecular level and thus can help discover the rules of cellular activities including growth, development, metabolism, differentiation and apoptosis [20]. The identification of protein interactions in a genome-wide scale is an important step for the interpretation of the cellular control mechanisms [21]. In this analysis, we selected DEGs with high degree of confidence and found out interacting object of the product of DEGs and constructed the PPI networks by prePPI [22].

Pathway enrichment analysis

Based on the deficiency of individual gene analysis, the gene set enrichment analysis evaluate differential expression patterns of gene groups instead of those of individual genes to distinguish whether the biological functions and characteristics changed [23]. In our analysis, the *P* value indicated the probability that a gene was endowed a Gene Ontology (GO) function randomly and it was usually used as the criterion for assigning a certain function to a module. GO function annotation, including Cytoscape [24], Mcode [25] and Bingo [26], was performed on these modules. The smaller the *P* value was, the more likely to prove that function of the module was not occurred randomly but for the purpose to accomplish a certain biological function, and it has unparalleled biological significance [27]. DAVID bioinformatics resources aimed at extracting biological meaning from large gene or protein lists systematically by the help of an integrated biological knowledge base and analytic tools [28]. The functional enrichment analysis for the screened DEGs was performed by DAVID, the FDR<0.05 was chosen as the cut-off criterion.
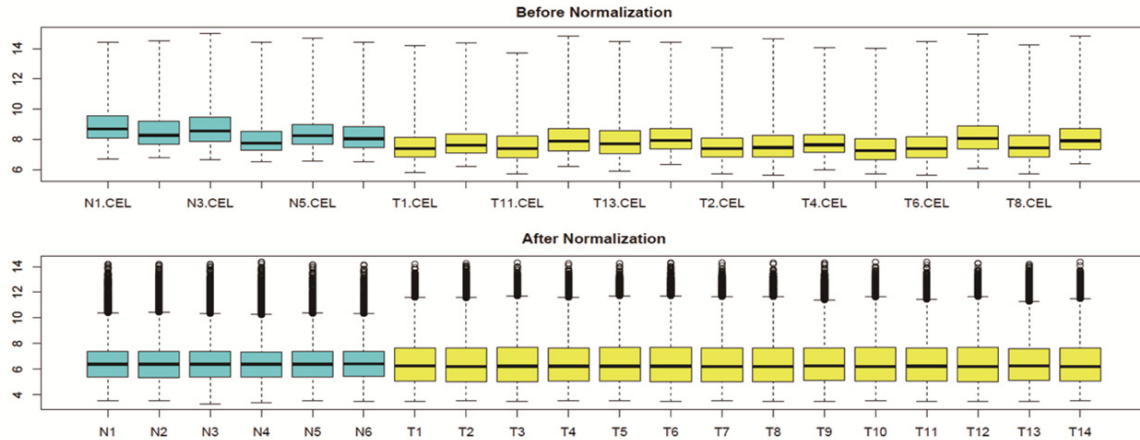
Figure 1. The box-plot of standardized expression data of normal tissue and OS tissue. The blue ones stand for normal samples while the yellow ones represents patients with OS. The medians of the samples were almost on the second dotted line, which indicated that the degree of standardization was very well.
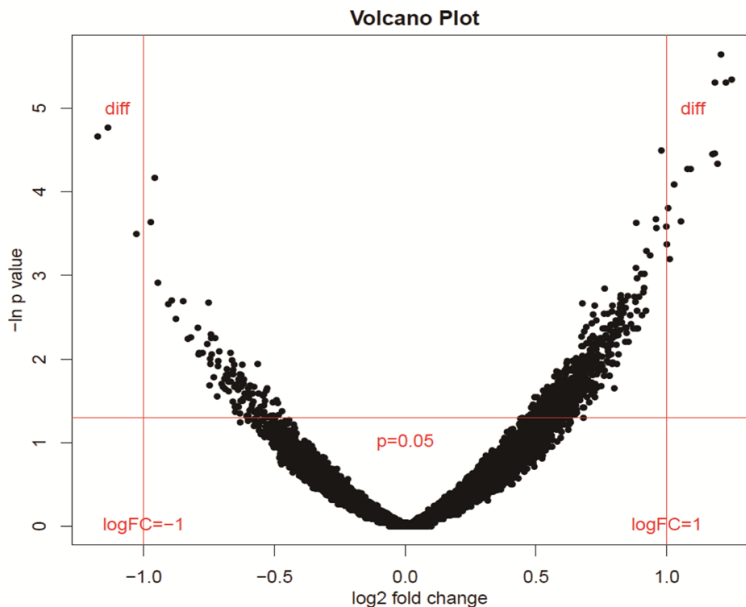

Figure 2. To verify the rationality of *P* value and corresponding fold change of expression data through different ways. The smaller *P* value is, the bigger the logFC will be, and then the figure will be shaped as zero, that's where the name volcano comes from.

at the molecular level and help discover the rules of cellular activities including growth, development, metabolism, differentiation and apoptosis [19]. The identification of protein interactions in a genome-wide scale is an important step for the interpretation of the cellular control mechanisms [15]. In this study, corresponding protein-protein interaction network of DEGs was constructed using the Cytoscape [20], based on the data collected from STRING online datasets. Besides, sub-networks were also established using MCODE (Molecular Complex Detection) [21] along with the extent of gene nodes. Then function analysis was performed on sub-networks later.

The pathway enrichment analysis of genes in the PPI networks where the up-and down regulated DEGs with maximal expression levels located was performed by using WebGestalt [29, 30]. The FDR<0.05 was selected as the cut-off criterion.

Protein-protein interaction network analysis

The protein-protein interactions (PPIs) [18] research could reveal the functions of proteins

Results

Data preprocessing

The standardized expression profiling data after preprocessing were shown in **Figure 1**, which can clearly show the difference before and after the standardization. The volatility of data after standardization is much stable than data before the process. For dataset GSE16088, a total of 1329 DEGs were identi-

Table 1. Differential expressed genes

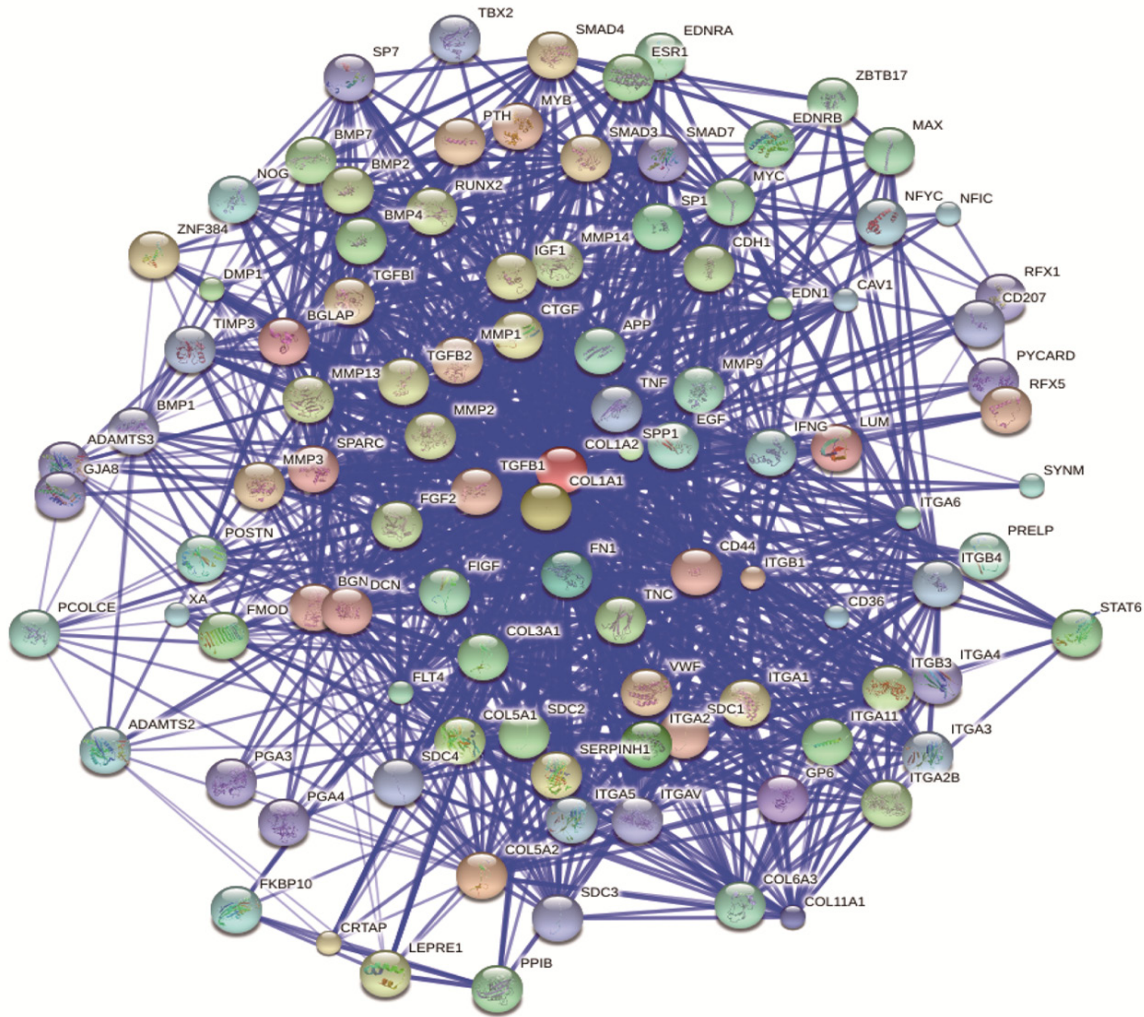| ID_REF	Gene Symbol	T test_adj	Wilcox_adj	Fisher_adj	logFC
201667_at	GJA1	1.11E-11	0.00054674	0.02724097	1.18443996
202404_s_at	COL1A2	6.05E-13	0.00137961	0.02724097	1.20747741
221729_at	COL5A2	1.23E-12	0.00137961	0.02724097	1.248982

Figure 3. PPI network. Protein information in different database was combined together by String. It can anticipate the interacting objects of target genes by the sequence, spatial structure of the protein.

fied at FDR<0.05 and |logFC|>1, including 648 down-regulated genes and 681 up-regulated genes. And the down- and up-regulated DEGs with maximum expression degree were UMOD gene and FABP7 (fatty acid binding protein 7) gene, respectively.

Differentially expression analysis

Only three DEGs were identified at *P*<0.05 and |logFC|>1 with the three statistic testing meth-

ods, along with multiple rectifying testing method **Figure 2**. Data is shown in **Table 1**. These genes will be used for further analysis.

Interaction network construction of DEGs

The PPI networks were constructed with the three selected genes at the core based on the data from STRING database, a tool which can combined protein information from several different database and can anticipate interaction

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- Coexpression -

association score 0.0 [gradient] 1.0

... from Coexpression in Homo sapiens:
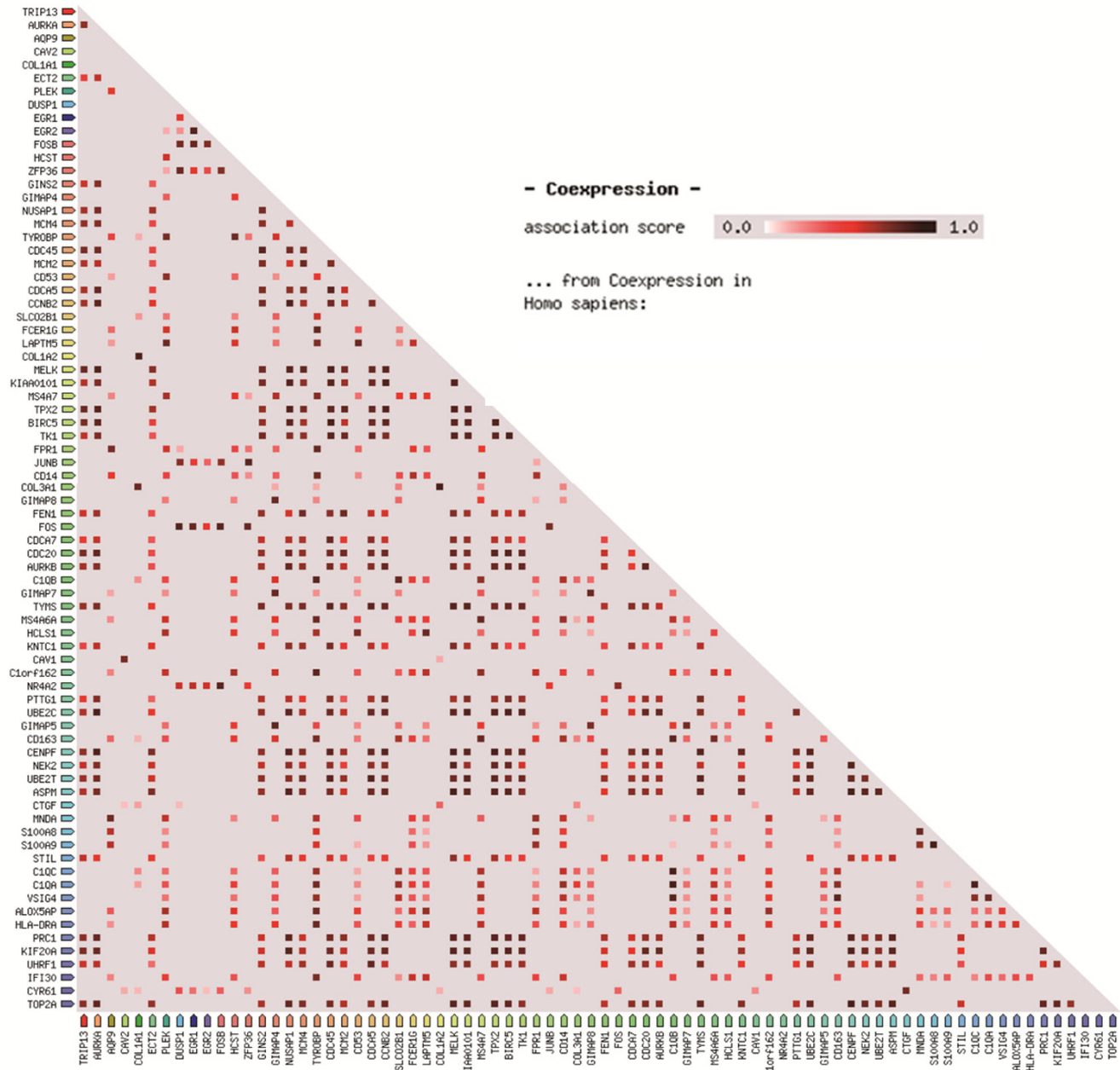
Figure 4. The co-expressing relationship map among the DEGs from the STRING database. The co-expressing degree is lower and lower with the dark red color becoming shallow gradually.
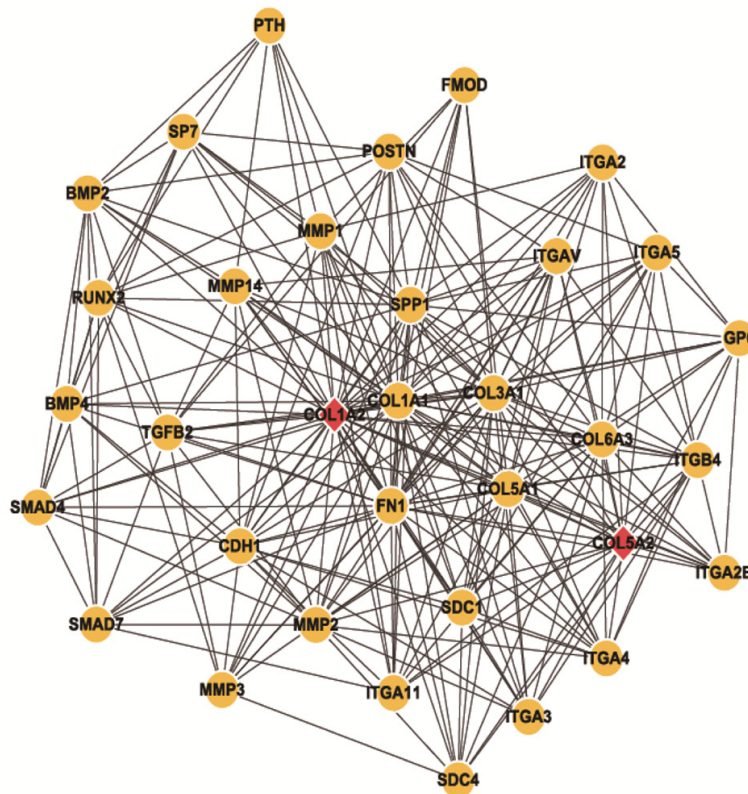

Figure 5. Co-expression module by Cytoscape and plug-in components. Yellow circles represent interacting genes while red rhombus represents the selected DEGs.

Table 2. Functional Enrichment analysis of DEGs in co-expression module

Term	Count	FDR
GO:0007067~mitosis	14	4.87E-08
GO:0000280~nuclear division	14	4.87E-08
GO:0000279~M phase	16	4.96E-08
GO:0000087~M phase of mitotic cell cycle	14	6.11E-08
GO:0048285~organelle fission	14	8.06E-08
GO:0022403~cell cycle phase	16	1.23E-06
GO:0000278~mitotic cell cycle	15	2.83E-06
GO:0007049~cell cycle	20	3.37E-06
GO:0051301~cell division	13	2.13E-05
GO:0022402~cell cycle process	16	8.18E-05
GO:0006260~DNA replication	10	4.57E-04
GO:0006259~DNA metabolic process	14	0.00107
GO:0010033~response to organic substance	16	0.00189
GO:0007059~chromosome segregation	7	0.00381
GO:0010564~regulation of cell cycle process	7	0.02769
GO:0009719~response to endogenous stimulus	11	0.03408

objects of specific known protein by analyzing protein sequence and spatial structure.

As shown in **Figure 3**, we found 207 and 44 interactive objects for UMOD gene and FABP7 gene according to the three-dimensional structure information of proteins included in prePPI software.

We mapped the DEGs to the STRING database and screened significant interactions with score larger than 0.8. By integrating these relationships, interaction network was constructed. The co-expressed relationship among the DEGs is revealed in **Figures 4** and **5**.

Gene ontology enrichment analysis

We used DAVID to analyze all the DEGs in the interaction network and found 16 significant function nodes. The information of DEGs in each function nodes is shown in **Table 2** and we can see that the most of them was related to cell cycle.

Module analysis

Module analysis was performed on the obtained network using Cytoscape software. Mcode was used to explore the mutual module of two genes and Bingo was used to do the annotation. The data was shown in **Table 3**.

Discussion

Tumors can be regarded as the abnormal expression of

Table 3. GO enrichment function analysis

GO-ID	Function	Corr *P*-value
7229	Integrin-mediated signaling	1.18E-12
48513	Organ development	1.18E-12
48731	System development	3.69E-10
22610	Biological adhesion	3.69E-10
7155	Cell adhesion	3.69E-10
48856	Anatomical structure development	3.24E-09
9888	Tissue development	5.69E-08
32501	Multicellular organismal process	7.84E-08
7275	Multicellular organismal development	7.84E-08
7166	Cell surface receptor linked signal transduction	0.000000135
7167	Enzyme linked receptor protein signaling pathway	0.000000168
7178	Transmembrane receptor protein serine/threonine kinase signaling pathway	0.000000194

the genetic materials of cell, so as the disease of osteosarcoma. Osteosarcomas are the most common primary malignant tumors of bone, showing complex chromosomal rearrangements with multiple gains and losses [31]. Residual tumor tissue as a result of unplanned excision of soft tissue sarcoma is a risk factor for local recurrence [32]. The extremely variable biological behaviour of malignant tumors is a diagnostic and prognostic challenge for clinicians and histopathologists alike [33].

In Jeon's study [32], twenty-five cases were selected to accept the tumor-removed surgery, which turns out that local recurrence rate increased and the whole survival rate without tumor was 65%. Local recurrence has lowered the patients' survival rate with an average of five or ten years [34]. Bramer [35] observed the level of alkalinephosphatase (AP) of eighty-nine adults cases before and after the chemotherapy, anticipated its relation with chemical reaction and survival. A pre-chemotherapy revealed AP was above twice Normal and correlated with worse survival, with the conclusion that a raised post-chemotherapy AP predicting poor chemotherapy response.

The target therapy for OS is becoming a popular subject in the clinical medical research, and the treatment for advanced OS has become an urgent issue giving its high occurrence rate on teenagers. Given the fact that there is not much research towards molecular inheritance of OS, in this study, we investigated the normal tissues and tissues of patients with OS to find out osteosarcoma-related genes in order to find out biomarkers of the disease. We analyzed differentially expressed genes (DEGs) in patients with osteosarcoma, in order to explore the relationship between the factor of heredity and the occurrence frequency of OS. It can be subservient in providing possible evidence in genetics for the precautions, clinical diagnose and treatment of OS. According to the results, COL (collagen) was obviously up-regulated among DEGs. We anticipated that it can be regarded as one of the feature genes in diagnose of OS.

The formation and rebuilt of bone are mainly associated with the diversion, proliferation of osteoprogenitor and cartilage progenitor cells and formation of extracellular matrix, a process precisely activated or surpressed by a series of genes. It has been proved that specific transcriptional factor of osteoblast plays an important role in regulating these genes, among which RUNX2 are the most important one [36, 37]. RUNX2 has been identified as a high risk factor and direct-related gene in osteosarcoma. However, there is rare research on other gene sequence associated with osteosarcoma. According to the results of cluster analysis in our study, corresponding genes of X17, X2, X4, X3, X18, X19, X12, X1, X5, X20, X7, X21, X6 may affect osteosarcoma in the same way as RUNX2 does, which may also belong to the genes of high risk factor and direct-related genes.

In this study, we are to explore the genetic factors effected on the prevalence mechanism of

osteosarcoma disease through analyzing the inter relationship between osteosarcoma disease related genes, and provide the genetic base more information on osteosarcoma for prevention, diagnosis and treatment of osteosarcoma. Though the research of gene therapy for tumor has a bright future, there are still different kinds of problems and difficulties. Different kinds of tumor have different pathogenic mechanisms, and some tumors are polygenic disease. We anticipated that COL as the possible feature genes of OS, but the molecular mechanism and target therapy of OS needs to be further explored and researched.

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Disclosure of conflict of interest

None.

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