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Identification of Key Gene Modules in Human Osteosarcoma by Co-Expression Analysis Weighted Gene Co-Expression Network Analysis (WGCNA)

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ABSTRACT

Osteosarcoma is the eighth-most common form of childhood cancer, comprising about 20% of all primary bone cancers. To date, systemic co-expression analysis for this cancer is still insufficient to explain the pathogenesis of poorly understood OC. The objective of this study was to construct a gene co-expression network to predict clusters of candidate genes involved in the pathogenesis of osteosarcoma. First, we contributed co-expression modules via weighted gene co-expression network analysis (WGCNA) and investigated the functional enrichment analysis of co-expression genes in terms of GO and KEGG. In result, seven co-expression modules were identified, containing 2,228 differentially expressed genes identified from the 22 human osteosarcoma samples. Subsequently, correlation study showed that the hub-genes between pair-wise modules displayed significant differences. Lastly, functional enrichment analysis of the co-expression modules showed that the module 5 enriched in progresses of immune response, antigen processing, and presentation. In conclusion, we identified essential genes in module 5 which were associated to human osteosarcoma. The key genes in our findings might provide the framework of co-expression gene modules of human osteosarcoma. Further, the functional analysis of these associated genes provides references to understand the mechanism of Osteosarcoma. J. Cell. Biochem. 118: 3953–3959, 2017.

KEY WORDS: OSTEOSARCOMA; CO-EXPRESSION; MODULES; GENE EXPRESSION; FUNCTION

Among a variety of bone and soft-tissue sarcomas, osteosarcoma (OS) is the most common primary bone malignancy with an overall incidence of 0.2–3/100,000 per year, and even higher incidence (0.8–11/100,000 per year) in the age group 15–19 years globally [Bielack et al., 2009; Mirabello et al., 2009]. Despite its rarity, it has been reported to be the third most common cancer in adolescence, occurring only less frequently than brain tumor and lymphomas in this age group. Usually, such peak incidence correlates with the pubertal growth spurt, occurring earlier in females than in males, and tall stature and high birth weight are proven risk factors [Mirabello et al., 2011]. Although the introduction of effective chemotherapy, including doxorubicin, high-dose methotrexate, cisplatin, and ifosfamide, has improved 3-year survival from 20% to 60–70%, no further improvements have been achieved in the last few decades [van Oosterwijk et al., 2013]. It should be noticed that better understanding of genetic etiology and pathology of OS may provide new possible treatment strategies for this tumor.

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Several small case-control studies have reported preliminary associations of common genetic variations with osteosarcoma risk in biologically reasonable pathways, such as TGFBR1*6A, which is a common mutation of TGF-β receptor 1, was reported to be associated with the distant metastasis of osteosarcoma [Hu et al., 2010]. Recently, Savage et al. [2013] suggested that two loci in the *GRM4* gene at 6p21.3 and in gene desert at 2p25.2, respectively may be involved in the mechanisms underlying susceptibility to osteosarcoma. However, only a handful of candidate genes are considered to be crucial in the pathogenesis of OS, and there is still large part needed to be explored.

Several computational approaches have been developed for the disease risk module analysis, which provide a significant measure for clinical prediction of cancer diagnosis and develop novel treatment strategies by calculating from a specific biological context [Hu et al., 2010; Zhao et al., 2011; Song et al., 2015; Wang et al., 2015a; Chen et al., 2016]. This includes detection of differentially correlated gene clusters and gene-specific analysis based the co-expression network [Bakhshi et al., 2009; Wang et al., 2015b], such as weighted gene co-expression network analysis (WGCNA) in R package. WGCNA is a powerful approach based on "guilt-by-association," it is used for identifying gene modules as candidate biomarkers or therapeutic targets based on the co-expression network [Langfelder and Horvath, 2008; DiLeo et al., 2011]. Comparing with many other analysis methods, WGCNA has an advantage to summarize and standardize the methods and functions for a comprehensive R package, including methods for both weighted and unweighted correlation networks. This systematical biology method has been used to study complex diseases, such as breast cancer [Clarke et al., 2013], schizophrenia [de Jong et al., 2012; Ren et al., 2015], and intracranial aneurysm [Zheng et al., 2015]. Besides, research has successfully utilized this method to identify pathway-related modules (for example, bladder cancer module) and hub genes (such as CAV1 and CCND1) in high-grade osteosarcoma [Ning et al., 2016]. With this reference, the route of co-expression network for models and hub genes of this cancer is accessible. However, relative research is still insufficient.

In this study, WGCNA was constructed basing on a data set comprising 2,228 genes from 22 human osteosarcoma samples. The correlation between each module and the biologic functions of genes detected in these modules are analyzed. These informative genes found in our study may be beneficial to clinical treatment of osteosarcoma. These genes and modules might be potential biomarkers for diagnose and treatment of high-grade osteosarcoma.

MATERIALS AND METHODS

DATA PROCESSING

Datasets for WGCNA related to osteosarcoma were obtained from the NCBI Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo) with accessing number GSE12512. The combined data set consist of a total of 22 samples. The samples were obtained from the tissues bank at the Laboratory of Oncology Research of the Rizzoli Orthopaedic Institute, Bologna, Italy. Prior to WGCNA analysis, we first mapped the array probes to the respective Gene ID by using the array annotations. Probes matching multiple genes were removed from the data set, and then we calculated the average expression values of genes measured by multiple probes. A proper threshold was settled based on the amount of genes filtered out.

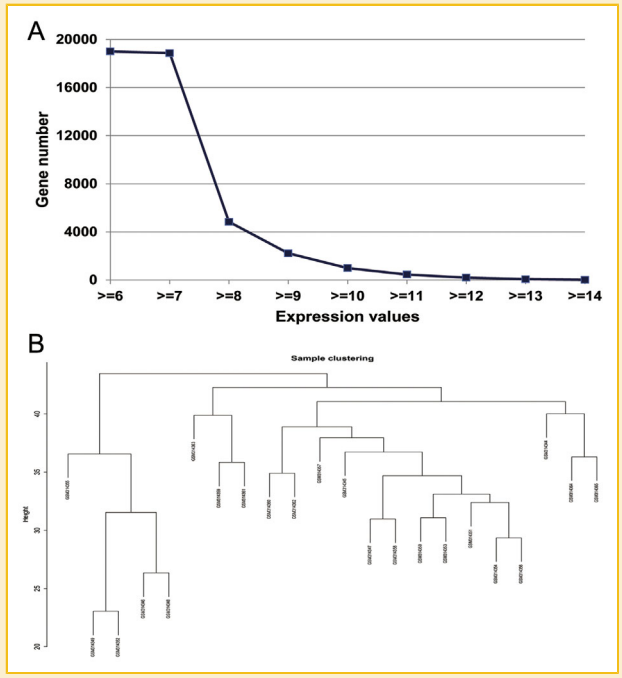

Fig. 1. (A) gene numbers corresponding to different gene expression thresholds. (B) Hierarchical clustering of module hub genes that summarize the modules yielded in the clustering analysis. Branches of the dendrogram (the meta-modules) represent together hub genes that are correlated positive.

CONSTRUCTION OF WGCNA TO IDENTIFY MODULES

In order to analyze the influence of power value on the scale independence and mean connectivity, we used the function *softConnectivity* from package WGCNA, with the "randomly selected genes" parameter set at 5000, other parameters set as default, and the power parameter pre-calculated by the *pickSoftThreshold* function of WGCNA. This function provides the appropriate soft-thresholding power for network construction by calculating the scale-free topology fit index for several powers. If the scale-free topology fit index for the reference dataset reaches values above 0.8 for low powers (<30), as the thresholding power defined in [12], it means that the topology of the network is scale-free and, therefore, that there are no batch-effects. We next summarized the expression values using the function collapseRows implemented in the R package WGCNA. Cluster analysis was subsequently performed by *flashClust* [Langfelder and Horvath, 2008]. The interactions (correlations) of each module was analyzed and visualized by heat map.

DETECTION OF HUB GENES AND THEIR FUNCTIONAL ANNOTATIONS

Hub genes are a loosely defined term for the abbreviation of "highly connected gene." That is, genes inside co-expression modules tend to

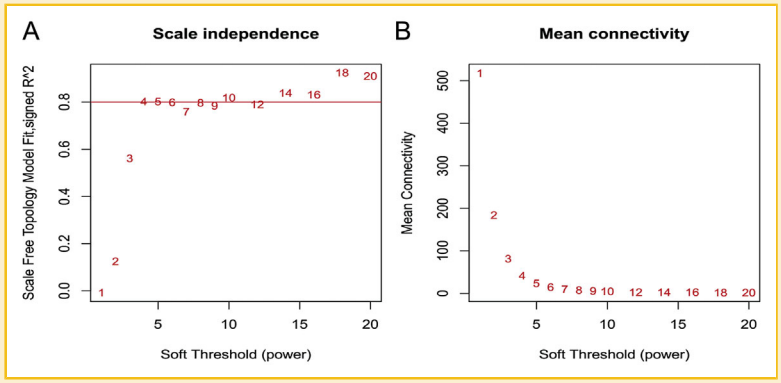

Fig. 2. Analysis of network topology for different soft-thresholding powers. The left panel (A) displays the influence of soft-thresholding power (x-axis) on scale-free fit index (y-axis). The right panel (B) shows the influence of soft-thresholding power (x-axis) on the mean connectivity (degree, y-axis).

have high connectivity will be selected as hub genes in the present study. We performed a gene ontology (GO) enrichment analysis for top five modules with most genes by using the Database for Annotation, Visualization, and Integrated Discovery (DAVID https://david.ncifcrf.gov/summary.jsp) [Dennis et al., 2003]. Functional enrichment analysis of the identified hub genes consisted of statistically highlighting the most overrepresented (enriched) GO terms and KEGG pathways ($P < 0.05$) [Ashburner et al., 2000; Kanehisa and Goto, 2000]. Functional annotation clustering combined single category with a significant overlap in gene content, and then assigned an enrichment score to each cluster in order to make interpretation of the results more straightforward.

RESULTS

DATA PRE-PROCESSING

To generate gene co-expression networks, the raw gene expression of osteosarcoma data sets were downloaded from the GEO data repository (http://www.ncbi.nlm.nih.gov/geo). The combined data set (GSE12512) contained a total of 22 classic OS samples, and the microarray platform is GPL7192. Raw data from each microarray dataset were pre-processed identically for background correction and normalization. Firstly, probes matching multiple genes were removed out from these data sets, and secondly the average expression value of gene measured by multiple probes was calculated as the final expression value. Finally, we identify

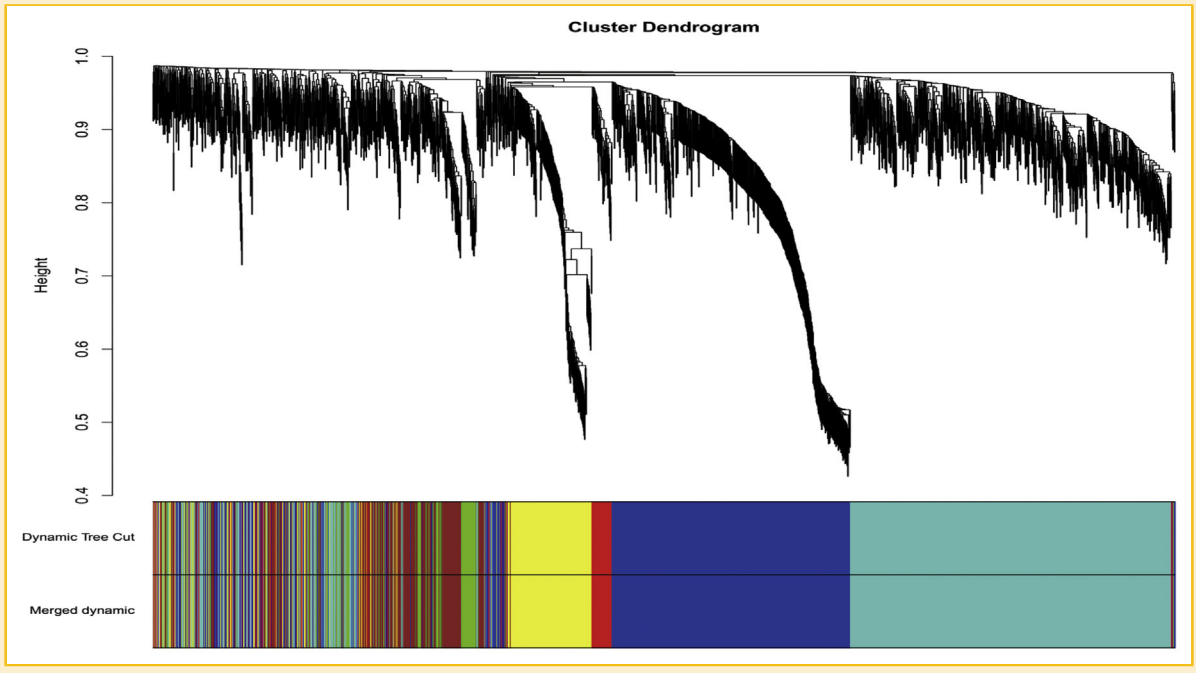

Fig. 3. Clustering dendrogram of genes, with dissimilarity based on topological overlap, together with assigned module colors.

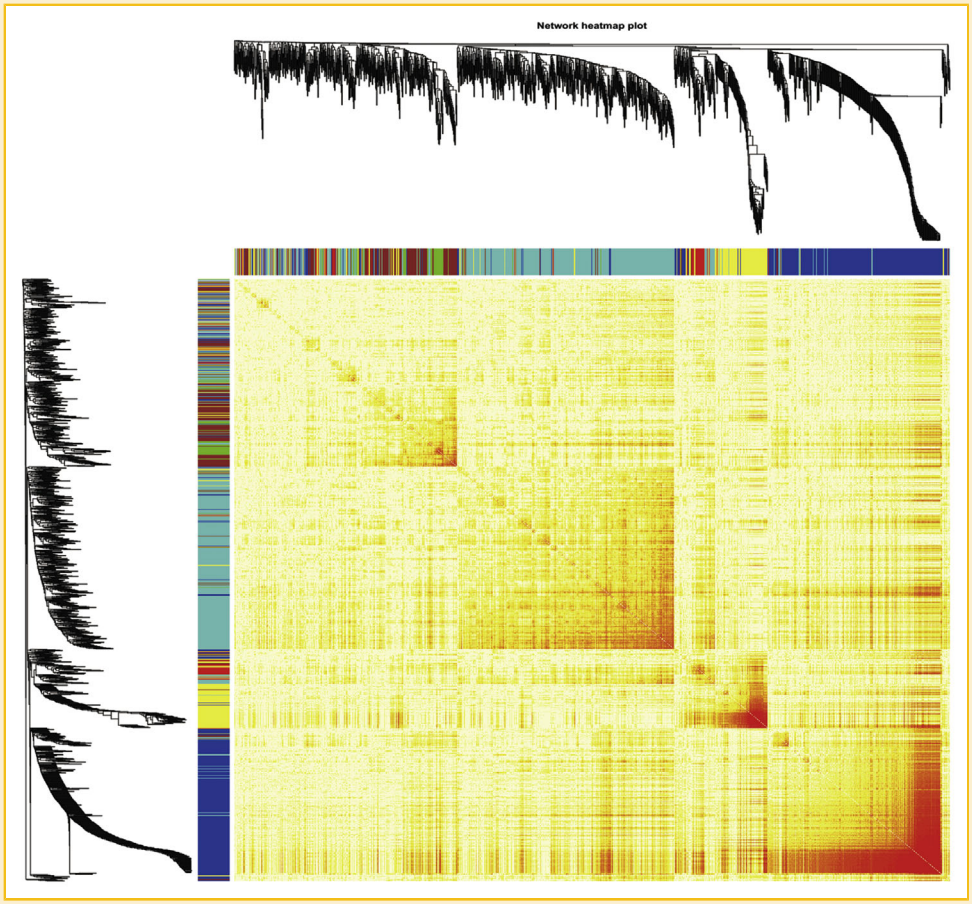

Fig. 4. Network heatmap plot. Branch in the hierarchical clustering dendrograms correspond to each module. Color-coded module membership is showed in the color bars under and to the right of the dendrograms. In this heatmap, the progressively more saturated yellow and red colors indicate the high co-expression interconnectedness. Modules correspond to highly interconnected genes blocks. Genes of high intramodular connectivity are located at the tip of the module branches because they show the highest interconnectedness with the rest of the genes in the module.

in total 19015 genes that were expressed. Hereafter, we plotted the relation of gene numbers and gene expression values (Fig. 1), and found that the lowest value is 6.9 and the highest is 14.8. Since the WGCNA was restricted to 3600 genes, we chose the the genes of which expression values are larger than nine. In total 2228 genes were filter out based on the requirement, which processed 11.7% of the total gene amount.

The 2228 genes were further investigated as input for hierarchical clustering analysis, which was performed with the function *flashClust*. We found that these 22 samples mainly yielded two clusters (Fig. 2A), where GSM314346, GSM314348, GSM314349, GSM314352, and GSM314355 became one cluster, the other 17 samples yielded the other one.

IDENTIFICATION OF GENE CO-EXPRESSION NETWORKS AND MODULES

Constructing a WGCNA needs the choice of the soft-thresholding power to which co-expression similarity is raised to calculate adjacency. Prior to WGCNA conducted to further study the 2228 genes obtained from the 22 samples discussed above, we first performed the analysis of network topology for various soft-thresholding powers in order to have relative balanced scale independence and mean connectivity of the WGCNA. As shown in Figure 2, power 4, the lowest power for which the scale-free topology fit index reaches 0.90, was chosen to produce a hierarchical clustering tree (dendrogram) of the 2228 genes (Fig. 3). Seven modules were generated and labeled 1–7 from largest to smallest. The largest module contained 838 genes while the smallest contains 318 genes, and averagely, each module contained 318 genes.

CORRELATION BETWEEN EACH MODULE

Based on the network heatmap plot (Fig. 4), each module showed independent validation to each other. Therefore, to further quantify co-expression similarity of entire modules, we calculate their eigengenes and cluster them on their correlation (Fig. 5A). These seven modules yielded two main clusters; one contained two modules, while the other contained the other five modules which can also be divided into three sub-clusters. This result was also supported by the heatmap plot of the adjacencies (Fig. 5B).

FUNCTIONAL ENRICHMENT AND CLUSTERING

A functional enrichment analysis was performed to examine the enrichment of annotated terms. Gene ontology (GO) enrichment

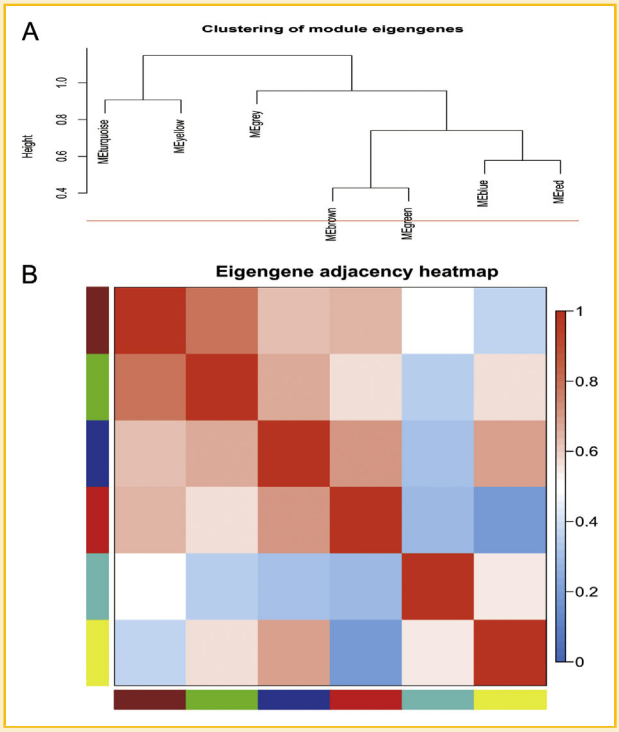

Fig. 5. (A) Hierarchical clustering of module hub genes that summarize the modules yielded in the clustering analysis. Branches of the dendrogram group together hub genes that are positively correlated. (B) Heatmap plot of the adjacencies in the hub gene network include the trait weight. Each column and row corresponds to one module hub gene (labeled by color) or weight. In the heatmap, red represents high adjacency (positive correlation), while blue color represents low adjacency (negative correlation). Squares of red color along the diagonal are the meta-module.

analysis for the first five largest network modules was performed by using the Database for Annotation (Table I), Visualization, and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov/). Supported by the network heatmap plot, each module had great difference with each other. Genes in modules 1 were mainly enriched in cell cycle and negative regulation of cellular protein, especially protein ubiquitination, module 2 and 4 were enriched in translation process, and module 3 was enriched in energy synthesis while module 5 was enriched in antigen processing and immune response.

To verify the result of GO enrichment analysis, KEGG pathways were analyzed on the same modules (Table II). The first four modules were enriched in proteasome (module 1), ribosome (module 2 and 4), and cell signaling and lysosome (module 4), while the module 5 was enriched in antigen processing, which is in consistence with GO result. Therefore, we speculated that the module 5, antigen process, and immune response, may play a key role in the pathogenesis of osteosarcoma

DISCUSSION

The main objective for this study was to utilize a global approach to construct a gene co-expression network that predicts clusters of candidate genes involved in the pathogenesis of osteosarcoma. We hypothesized that tightly co-expressed gene modules, enriched in shared functional annotation, would provide the most fruitful predictions of candidate gene sets that might underlie a given biological process.

WGCNA is a relatively novel statistical approach based on gene correlations, and has been used not only to construct gene networks and detect modules/sub-networks, but also to identify hub genes and select candidate genes as biomarkers [Langfelder and Horvath, 2008]. Usually, module detection in WGCNA needs a knowledge-independent process. However, selection of a threshold for culling the network to limit noise would probably rely on empirical judgment and functional annotation [Langfelder and Horvath, 2008]. Furthermore, WGCNA can only provide a set of hub genes instead of specific genes related to the background, such as osteosarcoma in this study. Therefore, further studies should be carried out to narrow down the gene targets. Such as

TABLE I. GO Enrichment Analysis in Co-Expression Modules

Term	Gene counts	Percent	*P* value	Benjamini
Module 1				
Cell cycle	66	0.8	1.30E-10	3.10E-07
Negative regulation of cellular protein metabolic process	28	0.4	2.80E-10	3.50E-07
Negative regulation of protein metabolic process	28	0.4	6.80E-10	5.60E-07
Negative regulation of protein ubiquitination	18	0.2	7.50E-10	4.70E-07
Anaphase-promoting complex-dependent proteasomal ubiquitin-dependent protein catabolic process	16	0.2	7.10E-09	3.50E-06
Module 2				
Translational elongation	51	0.8	4.20E-53	8.70E-50
Translation	61	0.9	2.60E-34	2.70E-31
Generation of precursor metabolites and energy	30	0.5	2.00E-09	1.40E-06
Oxidative phosphorylation	17	0.3	3.40E-09	1.70E-06
Ribosomal large subunit biogenesis	6	0.1	2.40E-06	1.00E-03
Module 3				
ATP synthesis coupled proton transport	6	0.3	9.40E-05	4.50E-02
Energy coupled proton transport, down electrochemical gradient	6	0.3	9.40E-05	4.50E-02
Module 4				
Translational elongation	14	0.7	1.40E-11	1.50E-08
Translation	18	0.9	2.00E-08	1.10E-05
Module 5				
Antigen processing and presentation	12	1.7	1.30E-13	8.60E-11
Antigen processing and presentation of peptide or polysaccharide antigen via MHC class II	8	1.1	1.50E-10	4.70E-08
Immune response	20	2.8	2.20E-10	4.70E-08
Antigen processing and presentation of peptide antigen	6	0.8	1.80E-07	2.90E-05
Antigen processing and presentation of exogenous peptide antigen	4	0.6	1.60E-05	2.10E-03

TABLE II. KEGG Pathways in Co-Expression Modules

Term	Gene counts	Percent	*P* value	Benjamini
Module 1				
Proteasome	14	0.2	3.90E-07	6.10E-05
Module 2				
Ribosome	46	0.7	1.80E-41	2.60E-39
Parkinson's disease	24	0.4	6.20E-10	4.50E-08
Oxidative phosphorylation	24	0.4	8.50E-10	4.10E-08
Huntington's disease	26	0.4	2.70E-08	9.90E-07
Alzheimer's disease	22	0.3	1.40E-06	4.00E-05
Module 3				
Epithelial cell signaling in Helicobacter pylori infection	9	0.4	1.80E-05	1.80E-03
Lysosome	11	0.5	2.80E-05	1.30E-03
Vibrio cholerae infection	8	0.4	4.30E-05	1.30E-03
Oxidative phosphorylation	9	0.4	1.70E-03	4.00E-02
Module 4				
Ribosome	14	0.7	2.80E-11	1.90E-09
Module 5				
Type I diabetes mellitus	10	1.4	3.30E-11	2.60E-09
Antigen processing and presentation	12	1.7	4.20E-11	1.60E-09
Viral myocarditis	11	1.6	2.00E-10	5.10E-09
Allograft rejection	9	1.3	3.30E-10	6.50E-09
Graft-versus-host disease	9	1.3	6.70E-10	1.00E-08

RMT method, this lies in its ability to automatically localize the noise-to-signal threshold instead of using empirical judgment or annotations [Ficklin et al., 2010]. Moreover, construction of mutant will also help to understand the role of one or more specific genes in pathogenesis of osteosarcoma.

Here, WGCNA was applied to investigate 2228 genes of 22 samples that were downloaded from a data set in NCBI. Finally, there were seven modules yielded. According to correlation study by network heatmap plot (Fig. 4), all the modules have almost no correlation with each other. GO enrichment and KEGG pathways analysis were performed to further study the biological functions of genes enriched in five largest modules. It was suggested that modules were involved in different functions/pathways (Tables I and 2). Module 1–4 were involved in protein ubiquitination, translation process, and energy synthesis etc; while the genes in module 5 were associated with antigen processing and immune system in both GO term and KEGG term.

In literature review, many previous studies suggested possible roles of genes involved in antigen and immune process in pathogenesis of OC as well. Endo-Munoz et al. [2010] have reported that OS are characterized by an early deregulation of genes involved in antigen presentation, and suggest that patient prognosis is determined early in tumor development and that enhancing antigen presentation may be clinically valuable in treating OS. Furthermore, several immune molecules, such as cytotoxic T cell lymphocyte antigen 4 (CTLA4) and CD40 (TNF receptor superfamily 5), have been targeted clinically in osteosarcoma to break immune tolerance to tumor [Paladini et al., 2016]. Findings of Tsukahara et al. [2004] suggested (*papillomavirus binding factor*) PBF as a shared tumor-associated antigen. It might serve as a source of peptide-based immunotherapy for osteosarcoma. 105AD7, a human monoclonal antibody, was also found over-expressed by osteosarcoma [Pritchard-Jones et al., 2005]. Therefore, antigen and immune process are essential and significant in pathogenesis of OC. The genes in module 5 may play a key role in the pathogenesis of osteosarcoma, and thereby provide potential targets for treating OS. Besides, as for there were still not enough research identifying many biomarker for OS, these genes in the present provided good references for further studies.

In summary, this study used transcriptional network analysis to identify a co-expression module for the first time. A module (module 5) was found highly enriched for genes involved in the antigen and immune process. And its collective expression was speculated to be correlated with pathogenesis of osteosarcoma. Data in current study were based on the expression profile downloaded from GEO database and the samples were relatively small hence, further extensive experiments need to perform to confirm results of this study. However, still, these genes in module 5 in our study might be potential biomarkers for diagnose and treatment of osteosarcoma.

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