Coexpression modules constructed by weighted gene co-expression network analysis indicate ubiquitin-mediated proteolysis as a potential biomarker of uveal melanoma

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Abstract. Uveal melanoma (UM) is a tumor that affects individuals throughout the world. Although gene expression analysis of UM has been performed previously, systemic co-expression analysis for this type of cancer remains lacking. Microarray data of UM samples was obtained from the Genome Expression Omnibus (dataset GSE44295). Co-expression modules were built by weighted gene co-expression network analysis. Functional enrichment analysis was performed on the co-expressed genes from important modules. Seven co-expression modules were constructed from the 5,000 genes gathered from the 58 human UM samples. The number of genes in these modules ranged from 73 to 3,051, with the mean number being 711. There was a marked difference in interactions among pairwise modules. Functional enrichment analysis demonstrated that module 2 was mainly enriched in pathways associated with the regulation of transcription. Additionally, modules 2-4 were significantly enriched in the ubiquitin mediated proteolysis pathway, suggesting it could serve a critical role in the occurrence and development of UM. The findings of the present study present a framework of co-expressed gene modules for human UM and provide an improved understanding of these modules at a functional level. Understanding the molecular mechanism and cellular pathways involved in pathogenesis of UM is extremely important for the development of more effective diagnostic and therapeutic strategies.

Introduction

Uveal melanoma (UM) is the most common type of malignant tumor that occurs in the eyes of adults (1). There are 6-7 cases/million people in America each year (2,3). Research on the gene expression module of UM is rare, which limits the understanding of critical genes associated with the occurrence and development of the disease. Despite the advances in the diagnosis and treatment of UM, the prognosis for patients with UM remains poor. UM frequently metastasizes and due to its hematologic nature, the liver is often the first site of metastatic disease. Approximately 50% of patients with UM will eventually succumb to their disease and the 5-year survival rate was 84% among all patients (4). Novel markers for UM are urgently required to improve the clinical management of individuals with the disease and increase their life expectancy.

Previously there have been few studies investigating the expression data of UM gene modules, which has limited the overall understanding of the function of critical genes within UM. Weighted gene co-expression network analysis (WGCNA) is a method frequently used to study biological networks with paired correlations between variables (5). WGCNA is a comprehensive collection of R functions for performing various aspects of weighted correlation network analysis (6). This technique has been widely used to study various biological processes, including cancer, genetics and brain imaging data, where it can help identify candidate biomarkers or therapeutic targets (7). Not only can WGCNA compare differentially expressed genes, but it can also discern the interactions between genes in different co-expression modules (8). WGCNA has been reported to have identified independent predictors of life expectancy for patients with breast cancer (7). WGCNA analysis has also been useful in the

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identification of potential oncogenetic drivers and therapeutic targets for patients with small cell lung cancer (9).

The present study aimed to construct co-expression modules using the gene expression data of patients with UM. Gene Ontology (GO) term and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed on the modules constructed, in order to establish the primary functions of the genes within these modules. These findings may be valuable for developing novel therapies to treat UM.

Materials and methods

Cluster analysis of UM microarray data. The microarray dataset GSE44295 and probe signal values were obtained from the Genome Expression Omnibus (GEO) database (ncbi. nlm.nih.gov/geo/query/acc.cgi?acc=GSE44295). This dataset comprised 58 samples of classic UM. The reference nos. of the UM samples were GSM1082563, GSM1082565-GSM1082566, GSM1082568-GSM1082575, GSM1082577-GSM1082584, GSM1082586-GSM1082596 and GSM1082598-GSM1082625. The sequencing platform used was GPL6883 HumanRef-8 Expression BeadChip (version 3.0; Illumina, Inc., San Diego, CA, USA). Microarray information was transformed into gene expression information by the expression value of probes from the GEO dataset. Microarray annotation information was used to match probes with corresponding genes. Probes with more than one gene were eliminated and the average value was calculated for genes corresponding to more than one probe. Samples with negative values were also eliminated. The threshold value was determined by the number of genes with a different threshold of expression. The WGCNA algorithm (7) was used to evaluate the gene expression value. The flashClust tools package (version 1.1.25; cran.r-project.org/web /packages/fastcluster/) in R language was used to perform the cluster analysis of the samples with appropriate threshold values.

Construction of co-expression modules for UM. The power value was screened out in the construction of each module using the WGCNA algorithm (7). The gradient method was used to test the independence and the average connectivity degree of different modules with different power values (the power value ranging from 1-20). The appropriate power value was determined when the degree of independence was 0.8. Once the power value was determined, the module construction proceeded using the WGCNA algorithm. The corresponding gene information for each module was extracted and the minimum number of genes was set as 50 to enable high reliability of the results.

Analysis of the co-expression modules. The interaction of co-expression modules was determined using R language (10) and the WGCNA algorithm. The Heatmap tools package (version 1.1.1; cran.r-project.org/web/packages/heatmap3) was used to analyze the strength of the interactions.

Functional enrichment analysis of the co-expression modules. The constructed modules were arranged by the number of genes they contained and then functional enrichment analysis was performed on the genes in these modules. The

corresponding gene information was mapped to the Database for Annotation, Visualization and Integrated Discovery (david. ncifcrf.gov/summary.jsp) (11). GO (12) term and KEGG (13,14) pathway enrichment analyses were then performed on the results. P≤0.05 after correction was used as the threshold for significant enrichment. The top five records were extracted if there were more than five significant results.

Results

Cluster analysis of UM samples. Following the elimination of probes with >1 gene, a total of 7,576 gene expression values were obtained. Then, the 5,000 genes with the highest average expression value were extracted. The flashClust tools package was used to perform the cluster analysis on these samples and the results are presented in Fig. 1. The 58 samples were divided into two clusters. A total of 38 samples were included in the first cluster, where sample GSM1082605 had the highest expression value. The second cluster included 20 samples, where sample GSM1082592 had the highest expression value.

Construction of co-expression modules for UM. The expression values of 5,000 genes in 58 samples of UM were used to construct co-expression modules using the WGCNA algorithm. One of the critical parameters was the power value, which mainly affected the independence and the average degree of connectivity within co-expression modules. Firstly, the power value was identified (Fig. 2). When the power value was 6, the independence degree was ≥0.8; the power value could appropriately assess the scale free topology of the network according to the WGCNA algorithm. Therefore, the power value used to construct the co-expression modules was determined to be 6.

As a result, seven co-expression modules were constructed (Fig. 3). These modules ranged from large to small by the number of genes they contained. The number of genes in the seven modules was 3,051, 529, 485, 453, 213, 173 and 73. The average number of the genes included in the seven modules was 711. There were 23 genes that did not belong to any of the seven modules.

Interaction analysis of the co-expression modules. Interactions of the seven co-expression modules were analyzed (Fig. 4). There was a clear difference between the interactions among different modules. The degree of connectivity between critical genes was analyzed in order to better clarify the interactions among the constructed co-expression modules (Fig. 5). With the exception of self-comparison, the strongest connectivity interactions were observed between modules 2 and 3, along with interactions between modules 2 and 7 (~0.8).

Functional enrichment analysis of genes in the co-expression modules. GO term and KEGG pathway enrichment analyses were performed on the genes in the seven constructed modules (Tables I and II, respectively). There was a significant difference in the biological processes that different modules were enriched in. Genes in module 1 were mainly enriched in translational initiation (GO:0006413), signal recognition particle-dependent co-translational protein targeting to membranes (GO:0006614) and translation (GO:0006412), while genes in module 2 were
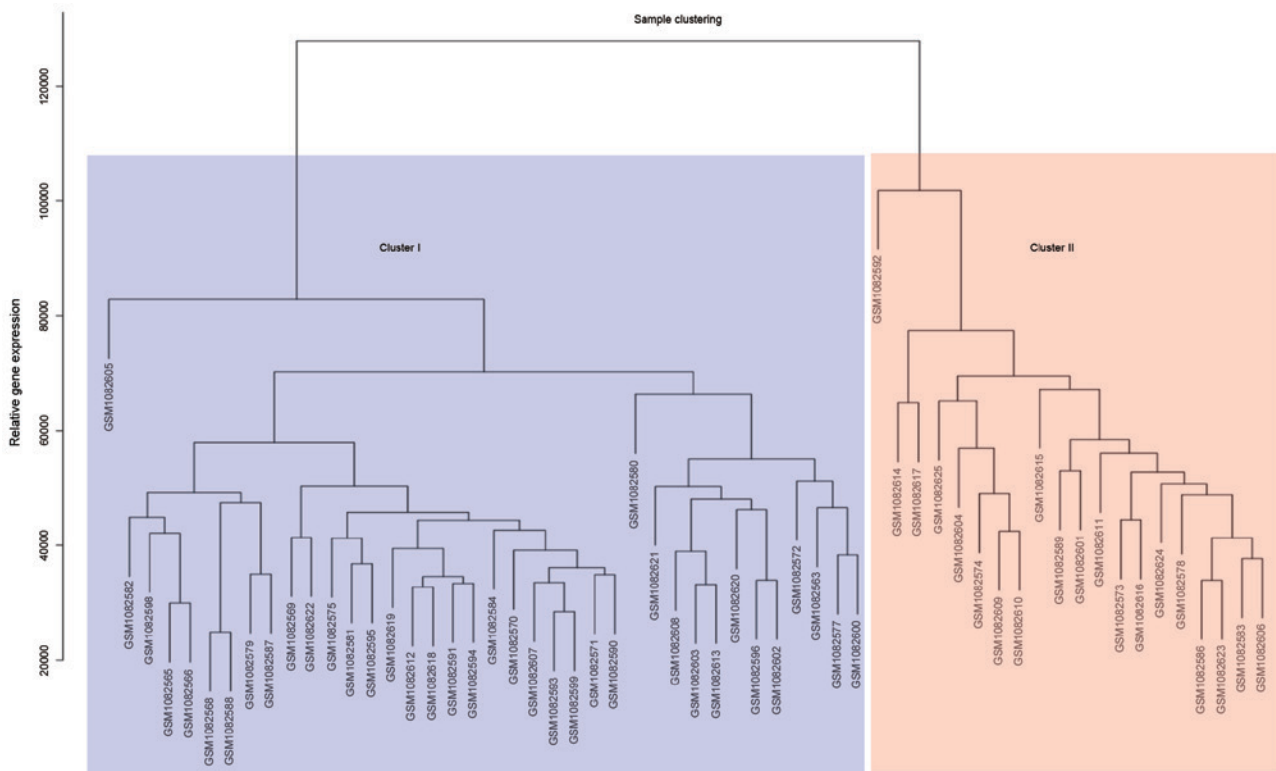
Figure 1. Cluster analysis of uveal melanoma samples. Cluster analysis was performed on the 5,000 genes with the highest average expression values by weighted gene co-expression network analysis and flashClust. All the samples were divided into two clusters, Cluster I (light blue) and Cluster II (light red), which included 38 and 20 samples, respectively.
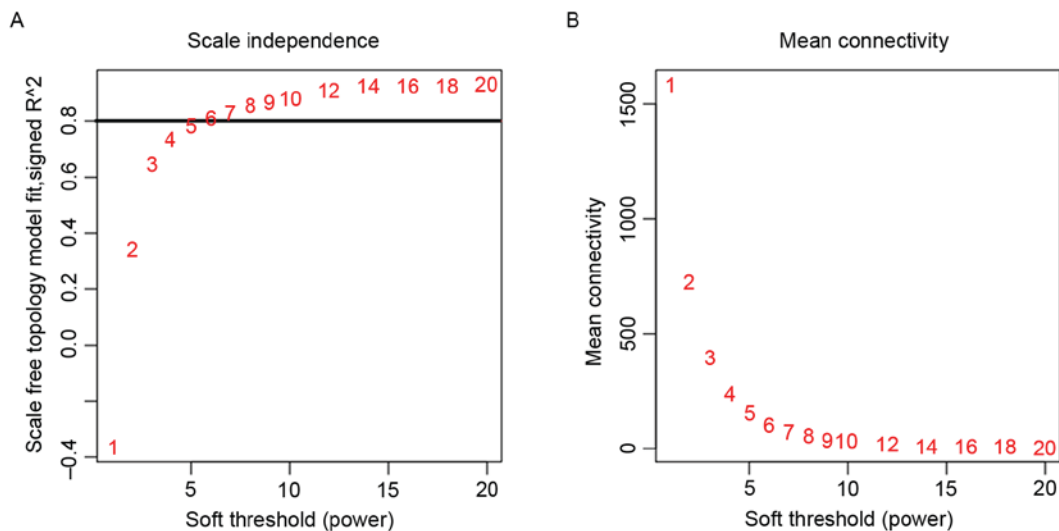

Figure 2. Power value screening of genes in UM co-expression modules. (A) The effect of different power values on the degree of independence between UM co-expression modules. (B) The effect of different power values on the degree of connectivity between UM co-expression modules. UM, uveal melanoma.

mainly enriched in transcriptional regulatory processes, including the regulation of DNA-templated transcription (GO:0006355), DNA-templated transcription (GO:0006351) and transcription from the RNA polymerase II promoter (GO:0006366). Genes in module 3 were mainly enriched in RNA processing and transportation processes, including RNA export from the nucleus (GO:0006405), mRNA splicing via the spliceosome (GO:0000398) and mRNA export from the nucleus (GO:0006406).

Genes in module 4 were similar to that of genes in module 3, which were mainly enriched in mRNA splicing via the spliceosome (GO:0000398). Genes in module 5 were mainly enriched in transcriptional regulation processes, such as the regulation of transcription from RNA polymerase II promoter (GO:0006357). Genes in module 6 were mainly enriched in biological processes associated with RNA processing and transcription, including RNA splicing (GO:0008380) and mRNA export from nucleus
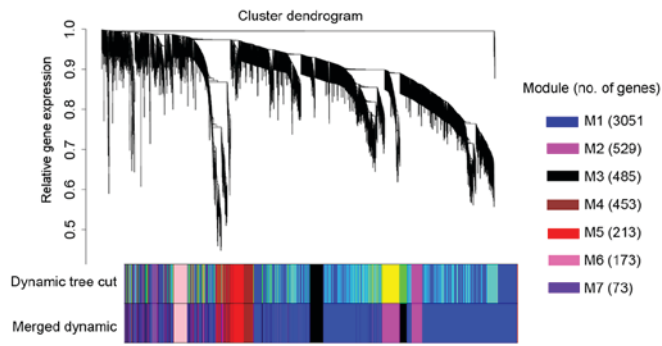
Figure 3. UM co-expression modules constructed. The WGCNA algorithm was used to construct co-expression modules for UM. Each branch represents a gene and the color below indicates each co-expression M. UM, uveal melanoma; WGCNA, weighted gene co-expression network analysis; M, module.
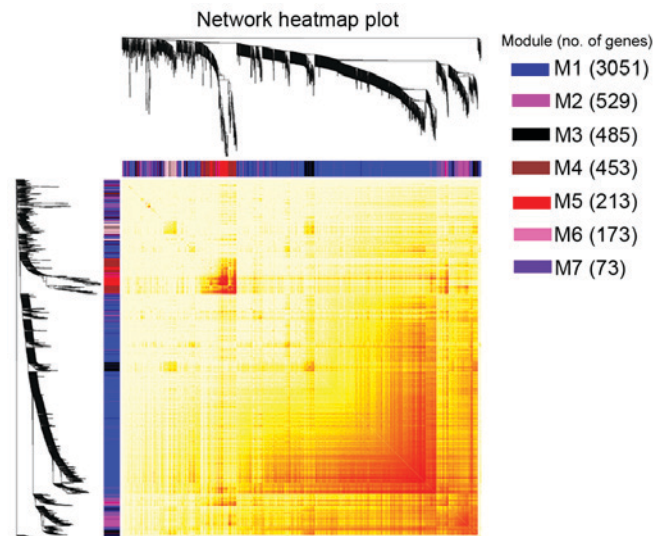

Figure 4. Interactions between genes in the co-expression modules. The different colors on the horizontal and vertical axis represent different modules. The yellow color in the middle represents the relativity among each module. There were notable differences in the correlation among different modules. M, module.
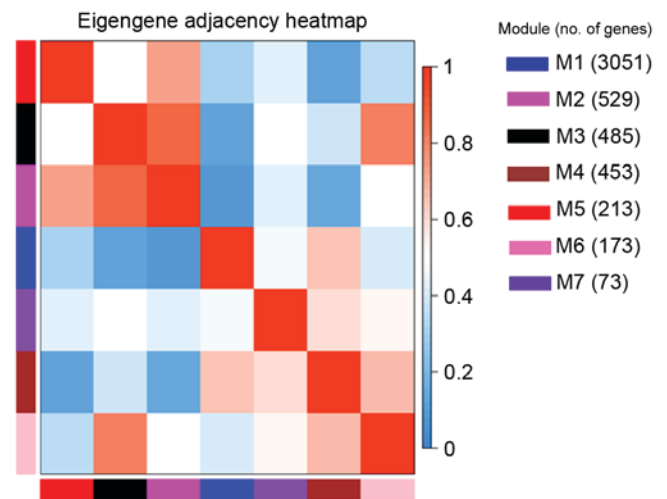

Figure 5. Connectivity analysis of critical genes in different modules. The color from blue (0) to red (1) in the heatmap represents the connectivity of critical genes in different modules from weak to strong, respectively. M, module.

(GO:0006406), which was similar to the result of modules 3 and 4. Genes in module 7 were mainly enriched in biological processes involving immune responses, including the type I interferon signaling pathway (GO:0060337), the interferon-γ-mediated signaling pathway (GO:0060333) and immune responses (GO:0006955).

KEGG pathway enrichment analysis was conducted on the genes in the 7 modules (Table II). The results demonstrated that there were significantly enriched pathways within all of the modules except module 5. Genes in module 1 were mainly enriched in metabolic pathways such as the ribosome (hsa03010) and oxidative phosphorylation (hsa00190), while genes in module 2 were mainly enriched in the notch signaling pathway (hsa04330), the RNA degradation pathway (hsa03018), cancer progression pathways (hsa05200) and ubiquitin mediated proteolysis (hsa04120). Genes in module 3 were also mainly enriched in ubiquitin-mediated proteolysis (hsa04120), and pathways associated with RNA transportation and degradation. Genes in module 4 were mainly enriched in ubiquitin-mediated proteolysis (hsa04120), while module 6 was mainly enriched in metabolic pathways associated with RNA transportation and processing. Genes in module 7 were mainly enriched in pathways associated with antigen processing and presentation (hsa04612), and herpes simplex infection (hsa05168).

Module 2 was regarded as a critical module in the occurrence of UM based on the result of the GO and KEGG enrichment analyses. Additionally, the ubiquitin mediated proteolysis (hsa04120) pathway was significantly enriched in modules 2-4. This suggests that ubiquitination may serve an important role in the occurrence and development of UM.

Discussion

In the present study, a total of seven co-expression modules were constructed from the 5,000 genes from 58 human UM samples using the WGCNA method. The results of the functional enrichment analysis revealed that there was a significant difference in interactions among different modules, and that this was largely associated with their different functions. The results demonstrated that module 2 was enriched in pathways associated with transcriptional regulation processes, and was regarded as a key module

in the occurrence and development of UM. The ubiquitin mediated proteolysis pathway was identified to be significantly enriched in modules 2-4, suggesting that it may exhibit potential as a prognostic and predictive marker for the survival of patients with UM.

In the present study the WGCNA method was used to construct the seven co-expression modules, each of which included a series of genes with similar expression profiles. Genes within the same module were considered to be associated with each other by function. This method, unlike regular cluster analysis, where clusters are constructed based on the geometric distance of data, was advantageous since the analysis performed by WGCNA has biological significance, making the results more applicable.

Table I. GO term enrichment analysis of genes in the co-expression modules.

Module	GO no.	Term	No. of genes	Background genes (%)	P-value
1	GO:0006413	Translational initiation	86	2.842975	7.33E-33
	GO:0006614	SRP-dependent cotranslational protein targeting to membrane	64	2.115702	3.31E-27
	GO:0006412	Translation	114	3.768595	2.07E-26
	GO:0000184	Nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	70	2.31405	2.09E-24
	GO:0019083	Viral transcription	67	2.214876	6.48E-24
2	GO:0006355	Regulation of transcription, DNA-templated	66	1.26E+01	3.17E-04
	GO:0006351	Transcription, DNA-templated	76	1.45E+01	0.002697
	GO:0006366	Transcription from RNA polymerase II promoter	25	4.77E+00	0.0108
	GO:0000122	Negative regulation of transcription from RNA polymerase II promoter	31	5.92E+00	0.023932
	GO:0045944	Positive regulation of transcription from RNA polymerase II promoter	38	7.25E+00	0.043971
3	GO:0006405	RNA export from nucleus	9	1.86722	7.36E-05
	GO:0000398	mRNA splicing, via spliceosome	17	3.53E+00	2.00E-04
	GO:0006406	mRNA export from nucleus	11	2.28E+00	2.43E-04
	GO:0016032	Viral process	21	4.36E+00	3.31E-04
	GO:0051301	Cell division	21	4.36E+00	6.34E-04
4	GO:0000398	mRNA splicing, via spliceosome	15	3.36E+00	6.48E-04
	GO:0006886	Intracellular protein transport	14	3.14E+00	3.30E-03
	GO:0006099	Tricarboxylic acid cycle	5	1.12E+00	0.004148
	GO:0000184	Nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	9	2.02E+00	0.006867
	GO:0006541	Glutamine metabolic process	4	8.97E-01	0.010284
5	GO:0006357	Regulation of transcription from RNA polymerase II promoter	12	5.77E+00	0.00501
	GO:0045494	Photoreceptor cell maintenance	4	1.92E+00	0.005265
	GO:0045664	Regulation of neuron differentiation	3	1.44E+00	0.018871
	GO:0006351	Transcription, DNA-templated	29	1.39E+01	0.036089
	GO:0045893	Positive regulation of transcription, DNA-templated	11	5.29E+00	0.03745
6	GO:0008380	RNA splicing	11	6.36E+00	2.89E-06
	GO:0006406	mRNA export from nucleus	9	5.20E+00	3.46E-06
	GO:0075733	Intracellular transport of virus	6	3.47E+00	9.57E-05
	GO:0006409	tRNA export from nucleus	5	2.89E+00	1.91E-04
	GO:0010827	Regulation of glucose transport	5	2.89E+00	2.15E-04
7	GO:0060337	Type I interferon signaling pathway	16	2.19E+01	5.44E-23
	GO:0060333	Interferon-γ-mediated signaling pathway	12	1.64E+01	6.13E-15
	GO:0006955	Immune response	17	2.33E+01	1.28E-11

GO, Gene Ontology; SRP, signal recognition particle; tRNA, transfer RNA.

The GO functional enrichment analysis revealed that module 2 was mostly enriched in transcriptional regulatory processes, including the regulation of DNA-templated transcription and transcription from the RNA polymerase II promoter. Module 2 was mostly enriched in KEGG pathways associated with metabolic processes, including the notch signaling pathway, the RNA degradation pathway, pathways in cancer and ubiquitin mediated proteolysis. Since the occurrence and development of cancer is accompanied by the abnormal expression of oncogenes or anti-oncogenes, the enriched pathways that module 2 is involved in may regulate the translation of protein-coding genes involved in the cell cycle or cell proliferation. Therefore, module 2 may be the most critical in the occurrence and development of UM.

The present study examined the degree of connectivity between critical genes to determine the interactions between

Table II. KEGG pathway enrichment analysis of genes in the co-expression modules.

Module	KEGG no.	Pathway	No. of genes	Background genes (%)	P-value
1	hsa03010	Ribosome	82	2.710744	6.68E-26
	hsa00190	Oxidative phosphorylation	63	2.082645	3.34E-13
	hsa05016	Huntington's disease	71	2.347107	8.71E-09
	hsa05012	Parkinson's disease	57	1.884298	1.14E-08
	hsa05010	Alzheimer's disease	64	2.115702	1.41E-08
2	hsa04330	Notch signaling pathway	7	1.335878	2.37E-03
	hsa03018	RNA degradation	7	1.335878	2.31E-02
	hsa05200	Pathways in cancer	19	3.625954	3.37E-02
	hsa04120	Ubiquitin mediated proteolysis	9	1.717557	4.37E-02
	hsa04141	Protein processing in endoplasmic reticulum	10	1.908397	5.47E-02
	hsa05169	Epstein-Barr virus infection	10	1.908397	9.76E-02
3	hsa04120	Ubiquitin mediated proteolysis	16	3.319502	2.50E-06
	hsa03040	Spliceosome	14	2.904564	4.08E-05
	hsa03018	RNA degradation	9	1.86722	8.54E-04
	hsa03015	mRNA surveillance pathway	8	1.659751	9.59E-03
	hsa03013	RNA transport	11	2.282158	1.46E-02
4	hsa04120	Ubiquitin mediated proteolysis	11	2.466368	2.30E-03
	hsa01100	Metabolic pathways	47	10.53812	2.54E-03
	hsa00020	Citrate cycle (TCA cycle)	5	1.121076	6.38E-03
	hsa01200	Carbon metabolism	8	1.793722	2.35E-02
	hsa03015	mRNA surveillance pathway	7	1.569507	2.68E-02
6	hsa03013	RNA transport	7	4.046243	0.002524
	hsa03040	Spliceosome	5	2.890173	0.022281
	hsa05202	Transcriptional misregulation in cancer	5	2.890173	0.046592
7	hsa04612	Antigen processing and presentation	12	16.43836	1.77E-13
	hsa05168	Herpes simplex infection	14	19.17808	9.17E-12
	hsa05150	*Staphylococcus aureus* infection	8	10.9589	1.51E-08
	hsa05332	Graft-vs.-host disease	7	9.589041	2.14E-08
	hsa05416	Viral myocarditis	8	10.9589	2.23E-08

KEGG, Kyoto Encyclopedia of Genes and Genomes; TCA, tricarboxylic acid.

the co-expression modules. The results demonstrated that there was a significant difference in the interactions among different modules; this may be as a result of their different functions. The results identified that there was strong interaction connectivity between modules 2 and 3 and modules 3 and 6. It was also revealed that ubiquitin mediated proteolysis was significantly enriched in modules 2-4. Several studies have reported that ubiquitination is associated with the occurrence and development of cancer when accompanied by the dysregulation of oncogenes (15,16). Mutation of the cellular tumor antigen p53 gene has been reported to be associated with the occurrence of the majority of different types of cancer. The ubiquitination pathway regulates p53 tumor suppressor protein stability, localization, and functions in normal and cancerous cells (17). Therefore, it has been suggested that ubiquitination serves an essential role in the occurrence and development of UM, as it was the most enriched function according to the functional enrichment analysis, and it can disturb the expression of relevant proteins involved in the cell cycle and proliferation. Ubiquitination is an important post-translational protein modification that regulates a host of critical cellular processes. It has been previously reported that the inhibition of ubiquitination was very effective in the treatment of multiple myeloma (18). Proteasome-mediated degradation is a common mechanism by which cells renew their intracellular proteins and maintain protein homeostasis (19). Cellular activity is often initiated by enzymes, which are proteins that are closely associated with cell cycle processes. The results of the present study suggest that ubiquitin is able to regulate the cell system within UM by controlling the activity or degradation of the majority of proteins in the cells of the eye. Since the majority of patients with UM succumb to liver metastases (20), the reason for which is unclear, the enriched signaling pathway identified in the present study may help to clarify the metastasis of UM tumors (21,22). The inhibition of the ubiquitin mediated proteolysis pathway or the genes included in the co-expression modules may be an effective treatment for UM (15).

In summary, module 2 was regarded as the most critical module in the development of UM disease. The ubiquitin mediated proteolysis pathway was identified as being significantly enriched in three modules, meaning the constituent proteins could have potential as diagnostic and prognostic biomarkers of UM; however, further research is required to investigate this.

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Availability of data and materials

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Authors' contributions

QW and MY conceived and designed the study. XH, HY and DH performed the computational experiments. QW and MY wrote the paper. CJW and JL reviewed and edited the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

All authors declare that they have no competing interests.

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