·Basic Research·

Identify the signature genes for diagnose of uveal melanoma by weight gene co-expression network analysis

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Abstract

• AIM: To identify and understand the relationship between co-expression pattern and clinic traits in uveal melanoma, weighted gene co-expression network analysis (WGCNA) is applied to investigate the gene expression levels and patient clinic features. Uveal melanoma is the most common primary eye tumor in adults. Although many studies have identified some important genes and pathways that were relevant to progress of uveal melanoma, the relationship between co-expression and clinic traits in systems level of uveal melanoma is unclear yet. We employ WGCNA to investigate the relationship underlying molecular and phenotype in this study.

• METHODS: Gene expression profile of uveal melanoma and patient clinic traits were collected from the Gene Expression Omnibus (GEO) database. The gene co-expression is calculated by WGCNA that is the R package software. The package is used to analyze the correlation between pairs of expression levels of genes. The function of the genes were annotated by gene ontology (GO).

• RESULTS: In this study, we identified four co-expression modules significantly correlated with clinic traits. Module blue positively correlated with radiotherapy treatment. Module purple positively correlates with tumor location (sclera) and negatively correlates with patient age. Module red positively correlates with sclera and negatively correlates with thickness of tumor. Module black positively correlates with the largest tumor diameter (LTD). Additionally, we identified the hug gene (top connectivity with other genes) in each module. The hub gene *RPS15A*, *PTGDS*, *CD53* and *MSI2* might play a vital role in progress of uveal melanoma.

• CONCLUSION: From WGCNA analysis and hub gene calculation, we identified *RPS15A*, *PTGDS*, *CD53* and *MSI2* might be target or diagnosis for uveal melanoma.

• KEYWORDS: weighted gene co-expression network analysis; microarray data; gene ontology
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INTRODUCTION

Uveal melanoma is an aggressive cancer and can cause a blind and death from metastasis. About 50% of uveal melanoma patients develop metastases within ten years from diagnosis and their median survival is 5 to 7mo after confirming case of metastatic lesions [1]. Although the improvements in diagnosis and the development of more effective local therapies for primary tumors, the rate of metastatic death and poorly prognosis remains unchanged [2]. Previous study utilized the individual differential expression levels of gene or protein and clinic phenotype to understand the mechanism of uveal melanoma [3-5]. With the development of proteomic technology and microarray assay, the recent study turned to perform high through-put method to analyze the proteome and genome of uveal melanoma [6-8]. Although many studies identified many markers for progress of uveal melanoma, the relationship between expression levels of genes and clinic traits were unclear yet. The data of microarray would provide more information to study. The

Table 1 Clinicopathological characteristics of uveal melanoma patient samples

Accession No.	Gender	Age	Thickness	LTD (mm)	DFS months	Sclera	Met	Treatment	*mda-9*
GSM685471	M	78	7	6	67	Y	N	None	L
GSM685474	F	61	10	14	55	N	N	Proton	L
GSM685475	F	74	5	10	41	Na	N	Proton	H
GSM685522	M	70	16	17	55	N	N	None	H
GSM686961	M	60	9	11	52	N	N	None	L
GSM687001	F	82	3	2	20	Y	N	Proton	L
GSM686962	M	74	7	17	40	N	N	Proton	H
GSM686963	M	33	7.56	15	15	Y	N	None	L
GSM686984	F	65	NA	15	36	Y	N	None	H
GSM686987	F	76	5.3	12	44	Y	N	None	L
GSM686986	M	59	5.2	14	43	Y	N	None	L
GSM686990	M	51	NA	16	40	Y	N	None	L
GSM685650	M	85	12	16	48	Y	N	None	H
GSM685651	M	48	8	12	57	N	N	None	L
GSM685470	F	77	7	9	54	N	N	None	L
GSM686991	M	62	4	20	41	Y	N	None	L
GSM687002	F	55	11	13	48	N	N	None	L
GSM687004	F	71	NA	16	42	Y	N	Proton	L
GSM685473	M	84	14	25	21	Y	Y	None	H
GSM685523	M	74	6	13	33	N	Y	Proton	H
GSM685601	M	64	15	23	31	Y	Y	None	H
GSM686985	F	69	4.9	16	17	Y	Y	None	H
GSM686988	M	51	10.5	15	19	Y	Y	None	H
GSM686989	M	80	16	12	19	N	Y	None	L
GSM685603	M	61	6	11	31	N	Y	None	H
GSM685652	F	69	13	12	25	N	Y	None	H
GSM685602	F	74	7	9	18	N	Y	None	H
GSM685472	M	66	6	14	17	N	Y	None	L
GSM687003	F	42	10	12	51	N	Y	None	H

DFS: Disease free survival; NA: Not available; LTD: Largest tumor diameter; Met: Metastasis; *mda-9*: Expression of mda-9; L: Low expression; H: High expression.

high through-put data shows global view for understanding the cells or issue. As far, some microarray data of uveal melanoma has been collected by Gene Expression Omnibus (GEO) database [9]. We could construct a biological network in systems level from GEO database.

In this study, GSE27831 microarray data that contains 29 samples are downloaded from GEO database. For investigating the relationships between expression levels of genes and clinic traits, we applied Weighted Gene Co-Expression Network Analysis (WGCNA)[10,11]. That is a technique that has uncovered patterns of gene co-activity correlated to clinic traits and corresponding to functional pathways[12].

The result shows that the WGCNA identified seven modules and four modules significantly correlated with clinic traits. From network connectivity calculation, the hub gene in each module has been identified. We show that *RPS15A*, *PTGDS*, *CD53* and *MSI2* were hug genes and might play a vital role in clinic diagnose. In particular, *RPS15A* is closely associated with radiotherapy and *MSI2* is closely associated with largest tumor diameter (LTD). These two clinic traits are important to tumor diagnose and therapy.

MATERIALS AND METHODS

Dataset Collected Expression profiles of mRNA for uveal melanoma samples were collected from GSE27831 (from NCBI GEO databasehttp://www.ncbi.nlm.nih.gov/geo/) [8]. The microarray was used for screenings of GeneChip HumanGenome U133plus2 arrays (Affymetrix, Santa Clara, CA, USA). The mRNA dataset contain 29 unique samples from uveal melanoma patients. Data were preprocessed following the RMA procedure of Console software normalization (http://www.affymetrix.com/). The clinicopathological characteristics of patient samples are downloaded from the study by Gangemi *et al*[8]. The table of clinicopathological characteristics of patient samples is listed in Table 1.

Construct Co -expression Network The WGCNA is employed to identify the co-expression modules [10,12,13]. WGCNA is implemented in the R software package (http:// www.r-project.org/). Co-expression methodology is typically used for studying relationship between gene expression levels. WGCNA start from the level of thousands of genes, identifies modules of co-expressed genes, and relates these modules to clinical variables and gene ontology (GO) information. Modules are defined in an unbiased fashion and initially denoted by colors. Grey denotes background genes outside of modules. Highly connective module genes are represented and summarized by their first principal component, and it has been called the module eigengene or ME[11].

The data set used for network construction consisted of 29 Affymetrix HG-U133plus2 microarrays surveying gene expression with 54 675 probe sets. For computational reasons, network analysis was limited 4000 probe sets with greater variance (note: although some genes are represented by multiple probe sets and other probe sets are not fully annotated, for consistency we refer to probe sets as "genes" through this study). When evaluating the significance of the module correlations of WGCNA, we corrected the *P* values for the number of modules and the number of tested phenotypic traits. The network analysis was applied to uveal melanoma data set, a signed weighted network adjacency matrix is defined as:

$$a_{ij}=\left|\frac{1+cor(x_i, x_j)}{2}\right|^b$$

Where x_i and x_j represent the expression value of probes which are numeric vector whose entries report the β values across the individuals. Note that the adjacency a_{ij} is a number between 0 and 1 that is a monotonically increasing function of the correlation coefficient. The power b is a soft-thersholding parameter that can be used to emphasize high positive correlations at the expense of low correlations. A major advantage of weighted correlation networks is that they are highly robust with regard to the choice of b [10].

In co-expression network, the genes represent the nodes and the a_{ij} represent the edges. The value of a_{ij} represents the strength connectivity of the edges.

Gene Ontology Enrichment The annotations and functions of proteins were obtained from DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/home.jsp) [14,15]. GO terms assigned a Benjamini-Hochberg adjusted *P*-value of less than 0.05 by DAVID were deemed to be enriched over the background gene set.

Modules Membership Measure and Statistic Analysis Module-trait associations were estimated using the correlation between the module eigengene and the phenotype (clinic traits), which allows easy identification of expression set (module) highly correlated to the phenotype. For each

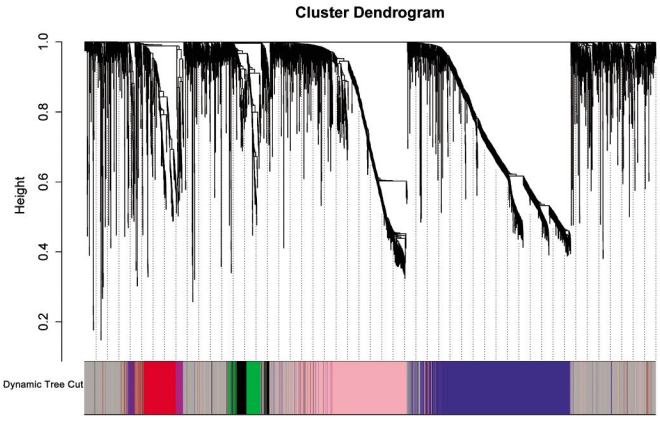

Figure 1 Network analysis of gene expression in uveal melanoma identifies distinct modules of co -expression genes. The dendrogram produced by average linkage hierarchical clustering of 4000 genes based on TO.

expression profile, gene significance (GS) was calculated as the absolute value of the correlation between expression profile and each trait; module membership (MM) was defined as the correlation of expression profile and each module eigengene. MM, also known as eigengene-based (eigengene: one of a set of right singular vectors of a genes x samples matrix that tabulates, *e.g.* the mRNA or gene expression of the genes across the samples) connectivity, is a measure of intramoduler connectivity. MM is defined as:

$MM(i)=cor(x(i), ME)$

Where $x(i)$ represents the expression profile of i th gene and ME represents the eigengene (first principal component) of the given module. We used the MM measure to select module genes for a GO enrichment analysis. For studying more significant association of MM, the threshold of $P<0.1$ was considered as significance value.

RESULTS

Constructing Gene Co -expression Networks in Uveal Melanoma We constructed gene expression networks from microarray data consisting of 29 uveal melanoma samples. All possible pairwise correlations were calculated for 4000 genes in uveal melanoma and converted into measures of connection strength by taking their absolute values and raising them to a power, β [10]. To identify modules of coexpressed genes, we searched for genes or high "topological overlap" (TO). We calculated TO and clustered genes on this basis for uveal melanoma. The results show that identifying seven distinct gene co-expression modules in uveal melanoma (Figure 1).

Gene Co -expression Modules Correspond to Clinic Traits The clinic traits are utilized another uveal melanoma study and the dataset was provided by GEO database. The clinic traits were listed in Table 1. And we removed some information that was useless in this study.

Sets of genes (modules) with common expression patterns

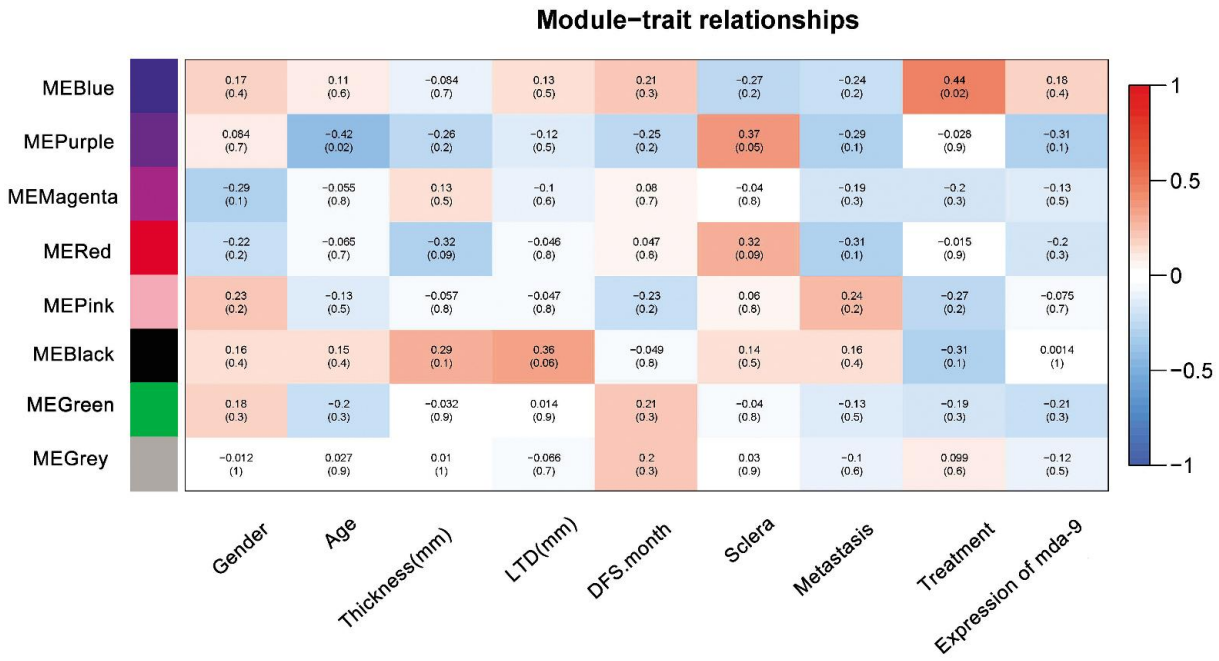

Figure 2 Correlation matrix of module eigengene values obtained for uvealmalanoma and clinic traits WGCNA groups mRNAs into modules based on patterns of their co-expression. Each of the modules was labelled with a unique color as an identifier. Eight modules were identified; each module eigengene was tested for correlation with clinic traits. Within each cell, upper values are correlation coefficients between module eigengene and the traits; lower values are the correspondent *P*-value.

that were associated with particular traits were identified based on the correlation between ME and clinic traits (Figure 2). We identified four modules that significantly associated with uveal melanoma clinic traits (P <0.1). Modules blue was correlated positively to treatment [$ME_{(blue)}$: treatment r=0.44, P=0.02]. Module purple was correlated positively to sclera and negatively to age [$ME_{(purple)}$: age r=-0.42, P=0.02; sclera r=0.37, P=0.05]. Module red was correlated positively to sclera and negatively to thickness [$ME_{(red)}$:sclera r=0.32, P=0.09; thickness r=-0.32, P=0.09]. Module black was correlated positively to largest diameter [$ME_{(black)}$: largest diameter r=0.36, P=0.06].

Gene Ontology Enrichment of Modules Associated with Clinic Traits The GO BP FAT function of DAVID to determine GO biological process enriched in the genes in all modules. Genes expressed in each dataset were set as the background measurement for DAVID. The Table 2 showed the GO analysis of modules that associated with clinic traits. The table showed the top 5 of GO biological process.

The GO results showed that the different modules were corresponded to different biological processes. From the Figure 2, we found that some traits have no correlated with any modules, such as gender, age, DFS, metastasis and expression levels of *mda-9*. In this study, we analyzed the four modules and tried to investigate the hub genes in each module network.

Inner Module Structure and Key Genes To determine genes that are centrally located in the modules that are associated with clinic traits, we calculated the intramodular connectivity. The intramodular connectivity (K-within) was calculated for each gene by summing the connection strengths with other module genes and dividing this number by the maximum intramodular connectivity. Genes with high intramodular connectivity are informally referred to as intramodular hub genes. The hub genes in the four modules were listed in Table 3.

The modules that closely associated with clinic traits might be very useful for uveal melanoma therapy and diagnose. The hub genes might provide signature target for further prognosis.

DISCUSSION

In this study we utilize gene expression data to identify genes involved in uveal melanoma. The WGCNA was employed to explore the relationship between uveal melanoma transcriptome and clinic traits. WGCNA has many advantages over traditional methods for differential expression analysis, including a focus on co-expression patterns thereby allowing for identification of biologically-relevant modules consisting of related genes, detection of hub genes that may eventually serve as targets for therapeutic modulation, and reducing data allowing for direct association analysis with disease-related variables. We identified 4 co-expression modules (gene networks) that relate to clinic traits status[16-20].

Previous study found that expression levels of *mda-9* relate with survival time and uveal melanoma metastasis. Unfortunately, we could not found any modules associated with expression levels of *mda-9*, metastasis and survival

Table 2 List of the top GO terms in the most significant DAVID functional clusters for each network module

Module	Top terms	No. of genes in ME	*P*	FDR
Blue	GO:0006414: translational elongation	1030	1.48e-48	2.54E-95
	GO:0006412: translation		2.88e-97	4.94e-49
	GO:0006091: generation of precursor metabolites and energy		1.59e-37	2.72e-34
	GO:0006119: oxidative phosphorylation		3.51e-36	6.02e-33
	GO:0022900: electron transport chain		4.51e-36	7.73e-26
Purple	GO:0030036: actin cytoskeleton organization	49	0.001889	2.673499
	GO:0030029: actin filament-based process		0.002387	3.366931
	GO:0007010: cytoskeleton organization		0.003335	4.674312
	GO:0006936: muscle contraction		0.005533	7.642884
	GO:0055002: striated muscle cell development		0.006677	9.154233
Red	GO:0009611: response to wounding	290	1.96E-20	3.32E-17
	GO:0006954: inflammatory response		7.91E-17	1.89E-13
	GO:0006952: defense response		1.90E-16	3.77E-13
	GO:0002504: antigen processing and presentation of peptide or polysaccharide antigen *via* MHC class II		8.16E-13	1.38E-09
	GO:0006955: immune response		1.40E-12	2.37E-09
Black	GO:0006355:regulation of transcription, DNA-dependent	119	1.10E-04	0.172753
	GO:0010629:negative regulation of gene expression		1.40E-04	0.220258
	GO:0051252:regulation of RNA metabolic process		1.54E-04	0.242911
	GO:0016481:negative regulation of transcription		2.92E-04	0.459812
	GO:0045449:regulation of transcription		4.00E-04	0.629367

FDR: False discovery rate; ME: Module eigengene.

Table 3 The hub genes of four modules

Module	Hub gene names	Uniprot accession	Description	Association traits	Intramodule connectivity (*k*)
Blue	RPS15A	P62244	40S ribosomal protein S15a	Radiotherapy treatment	284.57
Purple	PTGDS	P41222	Prostaglandin-H2 D-isomerase	Age and Sclera	7.88
Red	CD53	P19397	Leukocyte surface antigen CD53	Thickness and sclera	40.68
Black	MSI2	Q96DH6	RNA-binding protein Musashi homolog 2	Largest diameter	16.73

times. However, this study provided four modules correlation with other traits and would find novel therapy targets or prognosis targets for uveal melanoma.

The Table 2 shows the GO enrichment of the genes in modules. The module blue is associated with treatment. The treatment of this study is radiotherapy which utilizes proton beam. Therefore, the GO enrichment of module blue might reflect the irradiation response to uveal melanoma. The top 5 biological processes are mainly involved in translation and energy metabolism. Furthermore, the hub gene of module blue is *RPS15A* which is one of subunits of ribosome protein. In our previous study, we also found that the expression levels of energy metabolism genes were closely associated with radiation[6,7,21]. *RPS15A* is a highly conserved protein that promotes mRNA/ribosome interactions early in translation. Recent evidence showed that RPS15A could stimulate growth in yeast, plant and human lung carcinoma[22]. Another study indicates that *RPS15A* may play a key role in hepatic cancer cell growth [23]. Module blue contains many ribosome proteins which mainly were involved in protein synthesis. Module blue positively correlating with radiotherapy treatment indicates that irradiation promote up-regulated of translations and energy metabolism genes. *RPS15A* is a hub gene that strongly connects with other genes. So, *RPS15A* might be an important target of radiotherapy. Besides, *RPS15A* is also proved by recent research that *RPS15A* may play a prominent role in heptocarcinogenesis and serve as a potential therapeutic target in hepatocellular carcinoma[24].

The genes in module purple correlated with age and sclera. The GO analysis of module purple was mainly involved in cytoskeleton organization. And this module negatively correlates with and positively correlates with sclera. The hub gene is *PTGDS* that expressed in the eye and secreted into the aqueous humor (http://www.uniprot.org/uniprot/P41222). Therefore, we inferred that some tissue specific genes might highly express in uveal melanoma.

The genes in module red were mainly involved in inflammatory response and immune response. And the module red correlated with thickness of tumor and tumor location (sclera). The GO analysis and clinic traits indicated that inflammatory and immune response would relate with

thickness of tumor and tumor location. The hub gene *CD53* might contribute to cell survival in poorly vascularized regions of the tumor mass[25].

Module black is positively associated with LTD and the genes in this module are mainly involved in regulation of transcription. The function of hub gene *MSI2* is involved in regulation of the expression of target mRNAs [26]. The LTD is an important indicator for tumor relapse [27]. And the value of LTD is a predictor of survival after treatment of posterior uveal melanoma [4]. After conservative therapy, LTD is associated with increased risk of local tumor recurrence. In this study, we found the relationships between molecular level function and LTD. The regulation of transcription biological process might play a vital role in LTD. *MSI2* as a hub gene in black module might be a marker for LTD.

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