Comparative analysis of RNA-sequencing in intraocular tumors

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Abstract

Background: Intraocular tumors are a class of serious diseases which may cause blindness and disability. This study aims to explore the underlying mechanisms of gene regulation in intraocular tumors.

Methods: In this study, we firstly adopted RNA-sequencing technology to screen the differentially expressed genes in OCM-1 and Y79 cells compared with HRECs.

Results: We found 8424 differentially expressed genes (4665 downregulated and 3759 upregulated) in OCM-1 cells compared with HRECs cells, and 7486 differentially expressed genes (4250 downregulated and 3236 upregulated) in Y79 cells compared with HRECs cells. In addition, the GO (Gene Ontology) terms was used to predict the potential functions of these differentially expressed genes and KEGG (Kyoto Encyclopedia of Genes and Genomes) was used to analyze the potential functions of these genes in the pathways. Furthermore, we identified the Top differentially expressed genes by using qRT-PCR assay in intraocular tumors.

Conclusions: Here, we fully exposed the differentially expressed genes in intraocular tumor cell lines, which will improve understanding for gene regulation in intraocular tumors.

Background

Intraocular tumors can be divided into primary intraocular tumors and metastatic tumors[1]. The most common tumors were those located in the retina and uveal membrane, and scleral and optic nerve tumors were less common[1]. Intraocular tumor is a serious disease that causes blindness, which may endanger the life of patients[2]. After definite diagnosis, it is very important to choose appropriate treatment measures to preserve visual function and prolong life of patients. However, the incidence of intraocular tumors is relatively low. If the tumors are small and located in the peripheral omentum, there are often no obvious clinical symptoms[3]. Tumors involving the macular area or optic disc often cause irreversible visual impairment[4]. In particular, malignant tumors grow fast and easily invade adjacent tissues, and can reach all parts of the body through the blood and lymphatic system[5]. If it invades the brain nervous system, it will cause serious threat to the patient's life. Therefore, early diagnosis and effective treatment are extremely important for patients with intraocular tumors.

A transcriptome is the complete set of transcripts for a certain type of cells or tissues in a specific developmental stage or physiological condition[6]. A transcriptome analysis reveals the gene expression levels of organisms as well as structural variations and can be used to discover new genes[7]. The research methods and platforms for transcriptomes are undergoing rapid changes and bioinformatics analysis has also gradually improved. Over the past few years, the rapid development of second-generation sequencing (SGS) has also increased data throughput and read length, and simultaneously brought down sequencing costs[8]. This has allowed for new breakthroughs in the area of biology and ushered medical genetics into a new era. At present, advances in RNA-sequencing transcriptomics

methods have provided important research strategies for the transcriptional processes of various diseases[9], such as prostate cancer[10], breast cancer[11], Hirschsprung's disease[12], Alzheimer's disease[13], bladder cancer[14], sporadic Meniere's disease[15] and kidney disease[16], etc. However, the differentially expressed genes in intraocular tumors remain unclear.

In the present study, in order to identify the differentially expressed genes in different types of intraocular tumors, we detected, identified, and screened the differentially expressed genes by using RNA-sequencing technology in human retinal endothelial cells (HRECs), human uveal melanoma cells (OCM−1) and retinoblastoma cells (Y79). In addition, the GO (Gene Ontology) term was used to predict the potential functions of these differentially expressed genes, and KEGG (Kyoto Encyclopedia of Genes and Genomes) was used to analyze the potential functions of these genes in the pathways. In addition, we verified the Top differentially expressed genes in intraocular tumors by qRT-PCR assay. Finally, we obtained a global view of whole transcriptome alteration and provided precise molecular targets and signaling pathways, which contributes to better study for intraocular tumors.

Materials And Methods

Cell culture

HRECs was purchased from Angio-Proteomie (Boston, MA); OCM−1 and Y79 cells were all obtained from the type Culture Collection of Chinese Academy of Sciences (Shanghai, China). HRECs cells were grown in human retinal endothelial cell (HREC) medium including 10 mg/mL gentamicin, 0.25 mg/mL amphotericin B and microvascular growth supplements (MVGS; Invitrogen, Carlsbad, CA). OCM−1 and Y79 cells were cultured in the RPMI−1640 medium (HyClone Laboratories Inc., USA) containing 10% fetal bovine serum (FBS) 1% penicillin and streptomycin. All cells were maintained at 37°C in a cell incubator with 5% CO_2 and 95% air.

RNA extraction

According to the manufacturer's instructions, total RNA was extracted from HRECs, OCM−1 and Y79 cells by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). And a spectrophotometer was used to determine the absorbance at 260/280 nm.

Construction of cDNA libraries

cDNA library was prepared with 10 μg RNA per sample, and poly-(A)-containing mRNA was purified by using oligo (dT) magnetic beads (Thermo Fisher Scientific, MA, USA) and Oligotex mRNA Kits (Qiagen, Germany) following the manufacturer's procedure. The purified products were fragmented into small pieces, which were used as the template to synthesize first-strand cDNA with random hexamer-primers and reverse transcriptase. Then dNTPs, buffer, RNase H, and DNA polymerase were used to synthesize

the second-strand cDNA. After end repair process and ligation of adapters, the cDNA fragments were amplified using the PCR Master Mix. Finally, the library was quantitatively determined with the Agilent 2100 bioanalyzer instrument and RT-qPCR. The Illumina HiSeq 2000 System was (Illumina Inc., San Diego, CA, USA) used to qualify the libraries.

Analysis of differentially expressed genes

Single-cell RNA-sequencing data was normalized by using the Reads Per Kilo bases per Million reads (RPKM) method[17]. And then we filtered these genes by mapping algorithm. Genes above 10 reads were expressed after sequencing, but genes below 10 reads were not[18].

GO enrichment and KEGG pathway analysis

The hierarchical clustering was performed to determine the normalized expression level of each RNA type. The genes were input into the Gene Ontology Database (http://www.geneontology.org/) to execute GO annotation and enrichment analysis from three ontologies: molecular function, cellular component and biological process. The GO terms with the P-values ≤ 0.01 are defined as significantly enriched[19]. Furthermore, KEGG (Kyoto Encyclopedia of Genes and Genomes) database (http://www. genome.ad.jp/kegg/) was used to analyze the potential functions of the differentially expressed genes in the pathways. The genes with P ≤ 0.5 were considered significantly enriched.

Quantitative real-time PCR (qRT-PCR) assay

5 μg of total RNAs were reverse transcribed into cDNA through BestarTM qPCR RT kit (#2220, DBI Bioscience, China), and the quantitative polymerase chain reaction (RT-PCR) reaction was carried out via BestarTM qPCR MasterMix (#2043, DBI Bioscience, China) on an ABI7500 system. Primers used in this study were all synthesized in RiboBio (Guangzhou, Chnia). Relative gene expression levels were quantified by using $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

The data was presented as the mean ± standard deviation (mean ± SD). the results were counted by using SPSS 15.0 software (SPSS, Chicago, IL, USA). The data between two group was analyzed by Student's t-test; the data more than two group was assessed by variance (ANOVA); the data from GO enrichment was evaluated by Fisher's Exact Test. $P < 0.05$. was considered statistically significant.

Results

Analysis of transcriptional expressions in intraocular tumors

According to the RNA-Seq data, the reads per kilobase transcript per million reads (RPKM) distributions disclosed that the differential expression of each unigene in HRECs, OCM−1 and Y79 cells (*Figure 1A and 1B)..* The principal component analysis (PCA) of differentially expressed genes indicated that samples largely clustered based on cell type and culture conditions (*Figure 1C)..* In addition, the evaluation index of biological repeatability and correlation was assessed by Pearson's correlation coefficient analysis, and the results revealed the good biological repeatability and correlation in HRECs, OCM−1 and Y79 cells (*Figure 1D)..*

Identification of differentially expressed genes in intraocular tumors

The differentially expressed genes were identified by RNA-sequencing in HRECs, OCM−1 and Y79 cells (|log2 (FoldChange)| > 1 and fdr < 0.01). The volcano plot revealed the differentially expressed genes in HRECs, OCM−1 and Y79 cells, and the results showed that there were 8424 differentially expressed genes (4665 downregulated and 3759 upregulated) in OCM−1 cells compared with HRECs cells (*Figure 2A)*;; there were 7486 differentially expressed genes (4250 downregulated and 3236 upregulated) in Y79 cells compared with HRECs cells (*Figure 2B)..* In addition, a hierarchical clustering analysis then showed the differentially expressed genes in OCM1 cells compared with hREC cells (*Figure 2C)*,, and in Y79 cells compared with hREC cells (*Figure 2D)..*

The statistics of GO pathway enrichment in intraocular tumors

To understand more about the roles of differentially expressed genes in HRECs, OCM−1 and Y79 cells, the differentially expressed genes were assigned to GO terms, including biological processes, cellular components and molecular function terms. The typical enriched GO terms between OCM1 and hREC cells were shown *Figure 3A and Figure 3B,* the results indicated that the most enriched biological process terms are "single-organism cellular process", "single-multicellular organism process" and "developmental process"; the most enriched cellular component terms are "cytoplasm", "membrane" and "cytoplasmic part"; the most enriched molecular function terms are "binding", "protein binding" and "ion binding". Meanwhile, the typical enriched GO terms between Y79 and hREC cells were shown *Figure 3C and Figure 3D,* the results indicated that the most enriched biological process terms are "single-organism cellular process", "single-multicellular organism process" and "anatomical structure development"; the most enriched cellular component terms are "intracellular", "cytoplasm" and "cytoplasmic part"; the most enriched molecular function terms are "binding", "protein binding" and "ion binding". Therefore, we can summarize the most of the genes were located in the cytoplasm, related to single-organism cellular process, binding activity and other biological functions.

The KEGG pathway analyses of differentially expressed genes in intraocular tumors

KEGG database is a collection of various pathways, representing the molecular interactions and reaction networks. To identify signaling pathways involved in HRECs, OCM−1 and Y79 cells, the differentially expressed genes were submitted to Kyoto Encyclopaedia of Genes and Genomes (KEGG) analysis. We found that the differentially expressed genes were significantly enriched in 289 KEGG pathways. The top 20 enriched pathways were shown in *Figure 4.* Differentially expressed genes between OCM1 and hREC cells were highly clustered in several signaling pathways, such as "metabolic pathways", "regulation of actin cytoskeleton" and "PI3K-Akt signaling pathway". Differentially expressed genes between Y79 and hREC cells were highly clustered in several signaling pathways, such as "metabolic pathways", "PI3K-Akt signaling pathway" and "cytokine-cytokine receptor interaction". Therefore, we suggested that most of the genes were connected with metabolic pathways and PI3K-Akt signaling pathway.

Verification of the Top differentially expressed genes in intraocular tumors

To further confirm the differentially expressed genes in intraocular tumors, qRT-PCR assay was performed. The results revealed that SOX10, GAGE12F, ANXA6, GYPC and SLC45A2 expressions were significantly upregulated in OCM1 cells compared with hREC cells (P<0.05, P<0.001, *Figure 5A and Figure 5B)..* At the same time, we verified that FXYD6-FXYD2, APOBEC1 and SYT13 expressions were significantly upregulated in Y79 cells compared with hREC cells, PSG5 expression was significantly downregulated in Y79 cells compared with hREC cells (P<0.05, P<0.001, *Figure 5C and Figure 5D)..*

Discussion

Uveal melanoma and retinoblastoma are the most common intraocular malignancies in adults and children, respectively[20]. Uveal melanoma, a malignant cancer, can occur anywhere in the uveal tract, including choroid, ciliary body and iris, which affects 4.9 people per million in the United States[21]. Uveal melanoma is known for hematogenous spread, and the most common sites of metastasis were liver, lung, and bone from high to low[22]. Over the past few decades, the average five-year survival rate of uveal melanoma has held steady at about 80%, while the five-year survival rate sharply decreased when the tumor metastasizes[23]. Retinoblastoma is the most common primary intraocular carcinoma, affecting about 1/16,000 children worldwide[24]. Current treatments for retinoblastoma mainly include excision and topical treatment (laser, cryotherapy, chemotherapy or radiation)[25]. However, 5-year overall survival rate remains low in patients with advanced disease on account of local or distant metastases[26]. Therefore, exploring the potential molecular mechanisms of uveal melanoma and retinoblastoma may provide new therapeutic strategies.

Previous studies have shown the important roles of transcriptomes in various biological processes of uveal melanoma and retinoblastoma. For example, ADAM 10 could serve as prognostic factors for metastasis in primary uveal melanoma[27]; Pyrvinium pamoate inhibited the progress of uveal melanoma cells by downregulating Wnt/β-catenin pathway[28]; JSL−1, acts as a novel HDAC inhibitor, has anti-uveal melanoma activity[29]. MicroRNA−320 could suppress retinoblastoma progression by specificity protein 1[30]; long noncoding RNA BDNF-AS could be considered as a potential biomarker of retinoblastoma[31]; microRNA−433 suppress retinoblastoma progression by downregulating Notch1 and PAX6 [32]. However, the underlying molecular mechanisms in uveal melanoma and retinoblastoma have not been fully elucidated.

In recent years, it has been realized that the expression of individual genes in an organism is the result of a network of regulatory genes. Whether the changes in gene expression in uveal melanoma and retinoblastoma has not been clearly detected. Because of the characteristics of broad detection range and accuracy, RNA-sequencing has been widely used to identify large numbers of differentially expressed genes in organism. Based on the above research, we confirmed that the RNA-sequencing combined with OCM−1 and Y79 cells is an effective strategy to study the regulatory mechanisms of different genes. In the present study, HRECs cells as a comparison, differentially expressed genes in OCM−1 and Y79 cells were identified using RNA-sequencing. The data showed 8424 genes that were significantly dysregulated in OCM−1 cells compared with HRECs cells, and 7486 genes that were significantly dysregulated in Y79 cells compared with HRECs cells. In addition, we demonstrated that compared with hREC cells, SOX10, GAGE12F, ANXA6, GYPC and SLC45A2 were significantly upregulated in OCM1 cells; FXYD6-FXYD2, APOBEC1 and SYT13 were significantly upregulated in Y79 cells, which were consistent with the results in RNA-sequencing.

The GO and KEGG analysis were used to analyze the potential functions of these genes in the pathways[33]. It was found that altered expressions of genes included physiological functions such as single-organism cellular process, developmental process, cell adhesion, cell motility, cell migration, etc. the those that regulate corresponding signal transduction pathways, such as metabolic pathways, PI3K-Akt signaling pathway, MAPK signaling pathway, calcium signaling pathway, pathway in cancer, etc.

Conclusions

In summary, we comprehensively identified the differentially expressed genes in OCM−1 and Y79 cells compared with HRECs for the first time, and found 8424 and 7486 differentially expressed genes in OCM−1 and Y79 cells compared with HRECs, respectively. We also analyzed the annotations of the differentially expressed genes. In addition, our study is also the first to prove that SOX10, GAGE12F, ANXA6, GYPC and SLC45A2 were significantly increased in OCM1 cells; FXYD6-FXYD2, APOBEC1 and SYT13 were significantly decreased in Y79 cells. Therefore, our study systematically investigated the differentially expressed genes in intraocular tumors. This study may provide useful information for the treatment of intraocular tumors. Moreover, it will be necessary to explore functions and mechanisms of the certified genes in intraocular tumors.

Abbreviations

HRECs：human retinal endothelial cells

SGS：second-generation sequencing

KEGG: Kyoto Encyclopedia of Genes and Genomes

GO: Gene Ontology

 RPKM: reads per kilobase transcript per million reads

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no conflict of interest.

Funding

Authors' contributions

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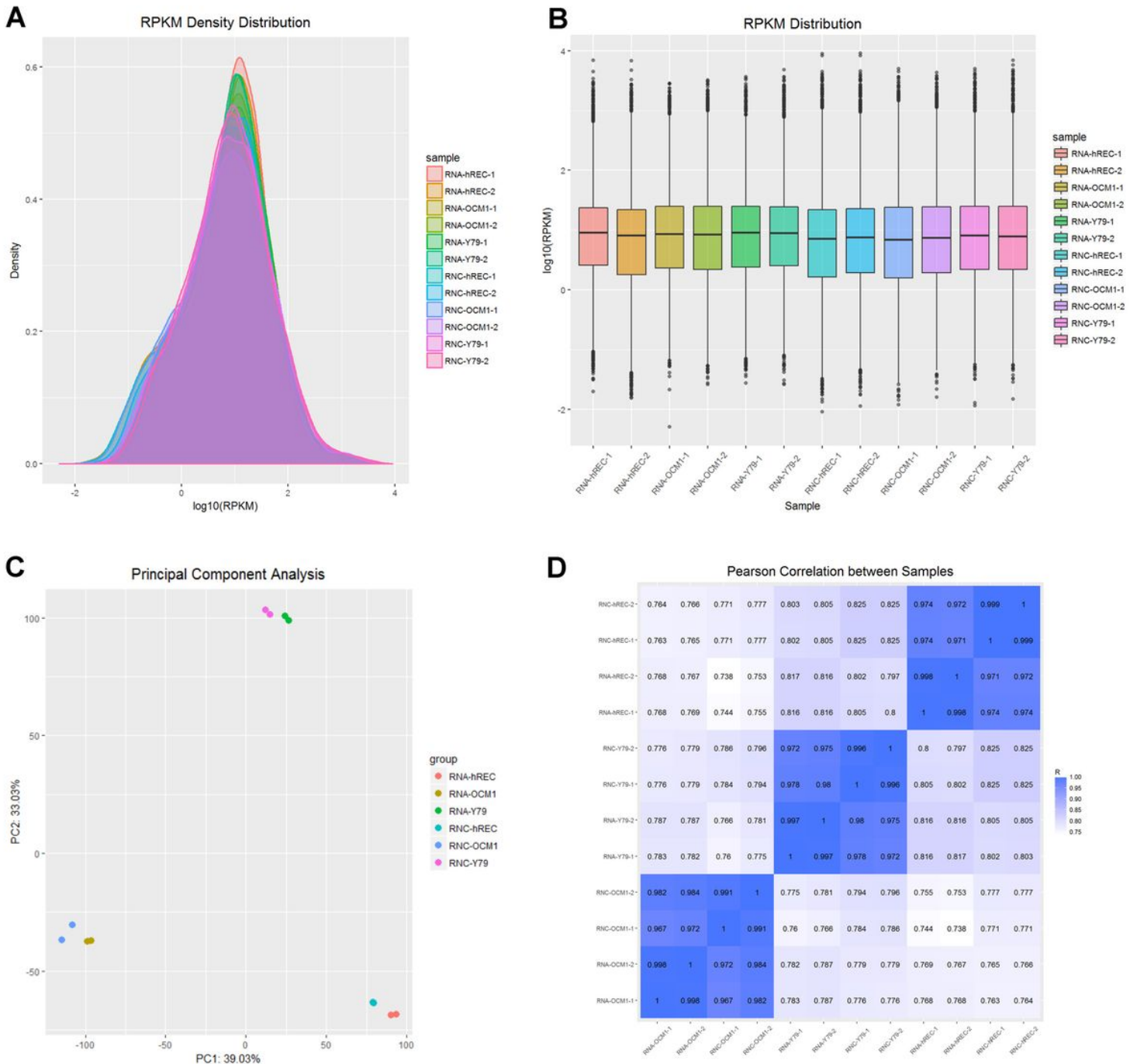
Figures

Figure 1

Analysis of transcriptional expressions in intraocular tumors. (A) The density distribution of FPKM in HRECs, OCM-1 and Y79 cells. The X-axis represents the distribution of RPKM. The Y-axis (left) represents the density of RPKM. (B) The boxplot of FPKM in HRECs, OCM-1 and Y79 cells. (C) The principal component analysis (PCA) of HRECs, OCM-1 and Y79 cells grown in distinct culture conditions, and one point shows the gene expression profile of a single cell. (D) The heatmap of the pairwise comparison in HRECs, OCM-1 and Y79 cells. The colored bar shows the expression levels of genes.
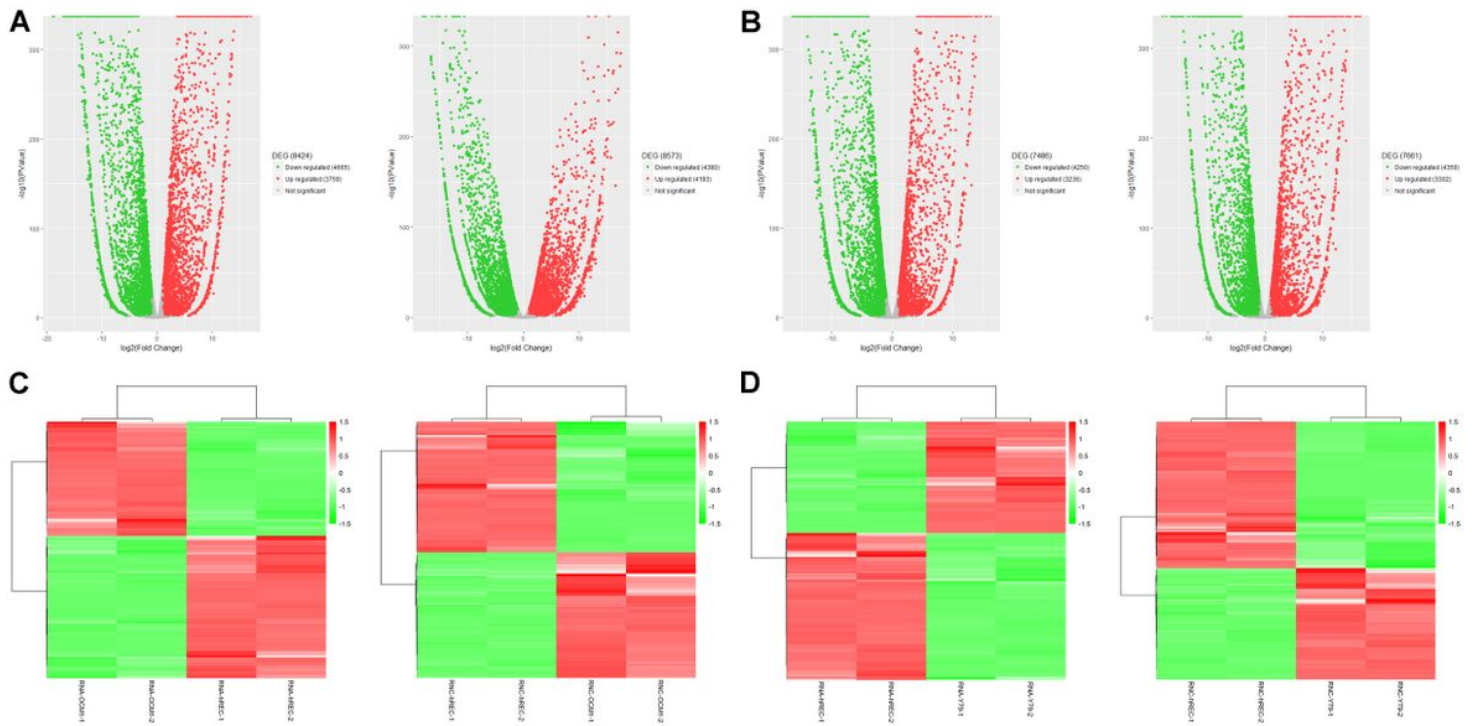
Figure 2

Identification of differentially expressed genes in intraocular tumors. (A) Volcano plot of the differentially expressed genes in OCM1 cells compared with hREC cells. (A) Volcano plot of the differentially expressed genes in Y79 cells compared with hREC cells. (C) A hierarchical clustering analysis of differentially expressed genes in OCM1 cells compared with hREC cells. (D) A hierarchical clustering analysis of differentially expressed genes in Y79 cells compared with hREC cells. Red indicates high expression genes and green indicates low expression genes.
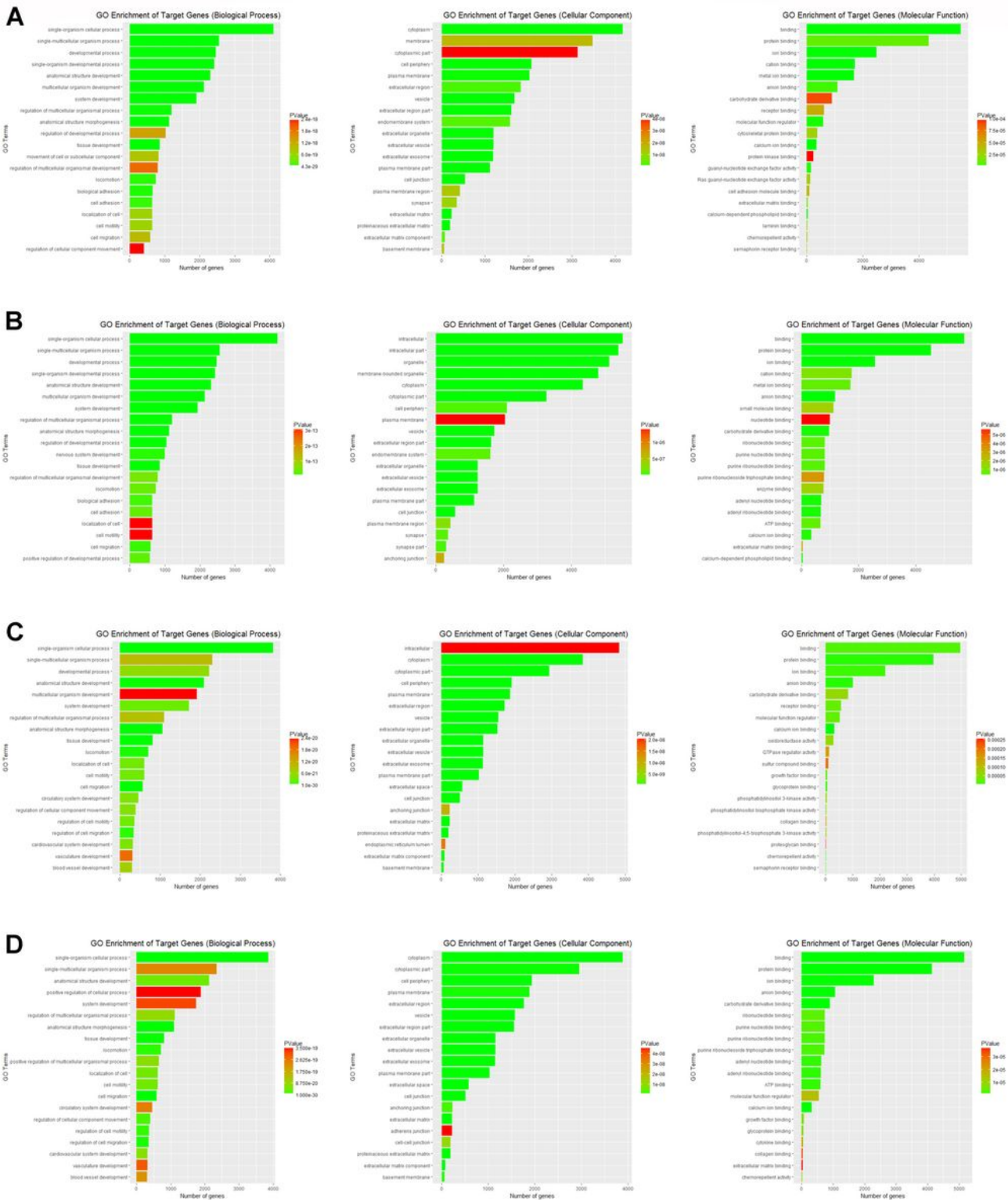
Figure 3

The statistics of GO pathway enrichment in intraocular tumors. The GO terms were shown in the Bar chart of biological processes, cellular components and molecular functions in RNA_OCM1 vs. hREC (A and B) and RNA_Y79 vs. hREC (C and D).
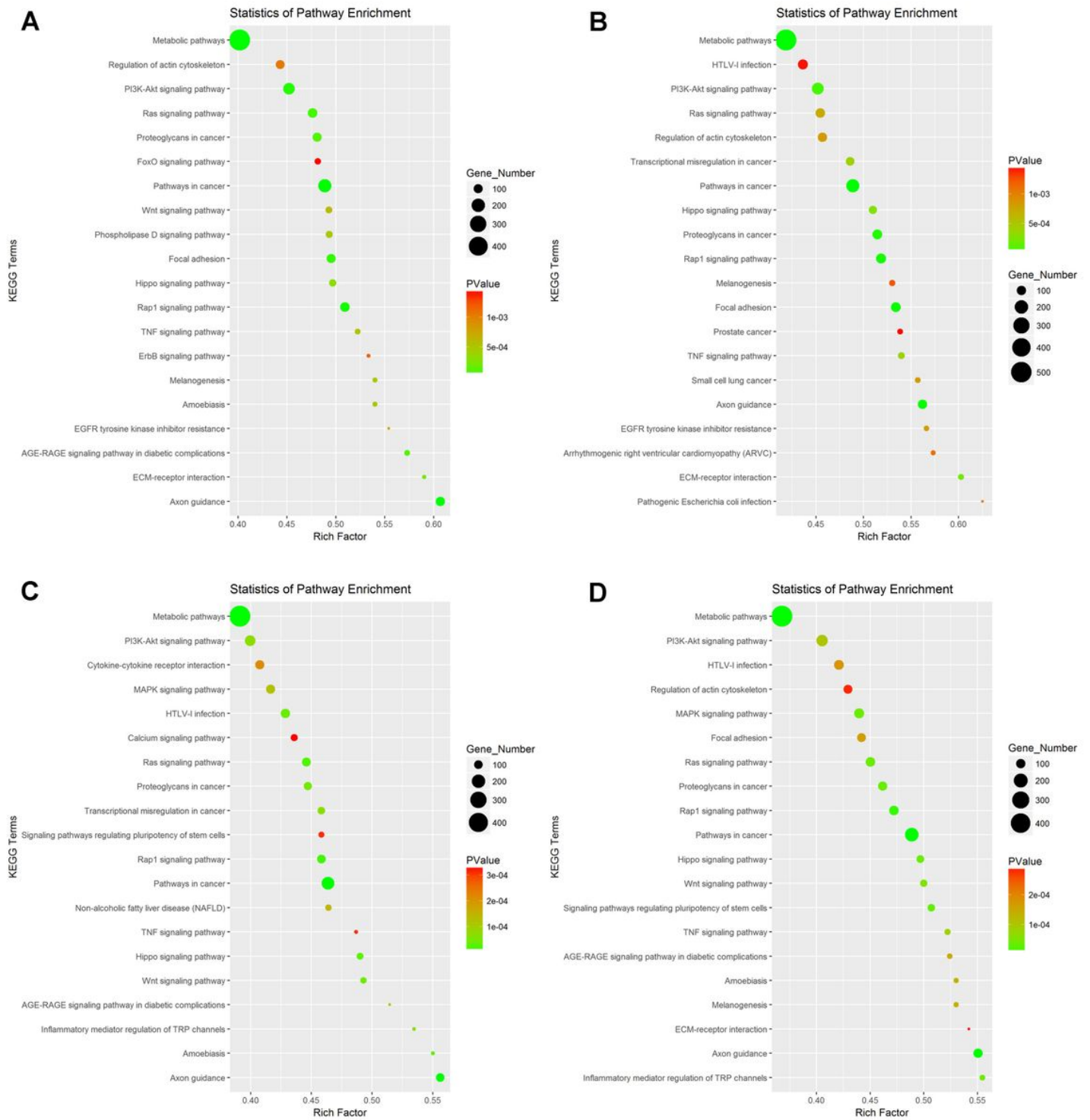
Figure 4

The KEGG pathway analyses of differentially expressed genes in intraocular tumors. Scatterplot of enriched KEGG pathway shows the top 20 pathways enriched in RNA_OCM1 vs. hREC (A and B) and RNA_Y79 vs. hREC (C and D).
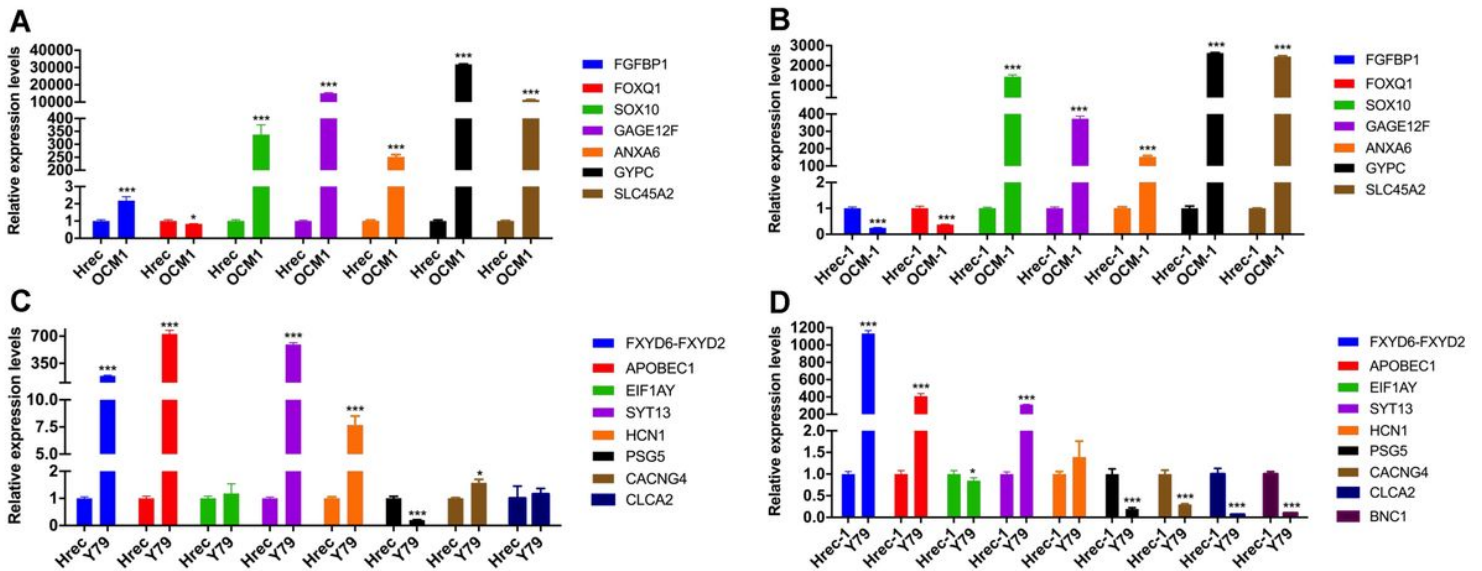
Figure 5

Verification of the Top differentially expressed genes in intraocular tumors. The mRNA expression levels of the Top expressed genes were analyzed by qRT-PCR assay in OCM1 cells compared with hREC cells (A and B) and in Y79 cells compared with hREC cells (C and D), *P<0.05, ***P<0.001 vs hREC cells group.