Altered expression of exosomal miRNAs from primary human trabecular meshwork cells induced by transforming growth factor- β 2

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Abstract

Background

Primary open-angle glaucoma (POAG) is tightly related with extracellular matrix (ECM) remodeling of human trabecular meshwork cells (HTMCs). Transforming growth factor- β 2 (TGF- β 2) can induce ECM remodeling. MicroRNAs (miRNAs) serve as a potential therapeutic target in influencing the development of glaucoma by regulating TGF- β 2. Recent studies also have found that exosomes may be involved in the regulation of intraocular pressure in patients of glaucoma. Therefore, the aim of the current study was to investigate the exosomal miRNAs expression changes derived from HTMCs treated with TGF- β 2.

Methods

Exosomes were isolated from HTMCs supernatant cultured for 24 h with TGF- β 2. The morphology of exosome pellets was examined by transmission electron microscopy. Nanoparticle tracking analysis used to demonstrate the particle size distribution. Total exosomal RNAs were extracted for subsequent miRNA gene chip analysis to investigate differentially expressed miRNAs between the control cells and treatment cells. Gene Ontology (GO) annotation, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were used to predict potential target and validate possible functions of the exosomal miRNAs.

Results

There were 23 miRNAs up-regulated and 3 miRNAs down-regulated. Go annotation and KEGG pathway enrichment analysis revealed that 469,102 and 94 GO terms involved in biological processes, cellular components and molecular function for the possible functions of the 26 miRNAs.

Conclusions

These findings indicate that TGF- β 2 may alter exosomal miRNAs expression to participate in the pathogenesis of POAG in which multiple molecular functions and pathways are involved. They may provide significant information for potential biomarkers for POAG diagnosis, clinical treatment and potential prognosis.

Background

Glaucoma is an irreversible ocular disease and the main cause of blindness around the world. It is characterized by optic neuropathy and corresponding visual field loss[1]. The most common form of glaucoma, primary open-angle glaucoma(POAG), is estimated to affect 58.6 million individuals by 2020[2]. Elevated intraocular pressure (IOP) is the only modifiable risk factor for POAG, presumably due

to increased resistance in the conventional outflow pathway including trabecular meshwork (TM) and Schlemm's canal[3, 4]. The pathophysiological processes leading to increased resistance in outflow pathways is yet to be fully elucidated. However, increased outflow resistance has been strongly correlated with aberrantly elevated levels of TGF- β 2 within the aqueous humor (AH) of POAG patients[5, 6]. Experiments *in vitro* and *in vivo* have shown that TGF- β 2 can enhance the synthesis and deposition of extracellular matrix (ECM) components; also, it inhibits TM cell proliferation and increases TM cell phagocytosis. Therefore, TGF- β 2 might play a vital role in the pathogenesis in POAG [7–9]. In this process, many genes altered expression is related to the ECM remodeling, but the exact regulation mechanism is unclear. Other researches have shown that TGF- β 2 could control miRNAs expression, which affected the metabolism of ECM in the TM[10–12].

Many miRNAs were found in TM tissue and AH. Some miRNAs expression may affect the TM function and the synthesis of ECM in different ways[13–15]. For example, miR-29 family functions as a critical suppressor of various ECM proteins in the TM and can be modified by TGF- β 2[12]. Some miRNAs maybe maintain homeostasis of IOP by regulating the target genes in TM[15–17]. However, the mechanism behind changes of miRNAs is unrevealed, and it is not clear how the miRNAs regulate gene expression or affect ECM function.

MiRNAs are found not only within but also outside of the cell, i.e., in the form of the exosome. Recent studies have demonstrated that exosomes may be involved in the regulation of IOP [18–22]. Exosomes are nanovesicles (30–150 nm in diameter). They are released into the extracellular environment by nearly all cells of the human body under both physiological and pathological conditions, including intraocular tissue cells[23, 24] and were detected in a number of body fluids, including AH, blood plasma, urine, saliva and breast milk[20, 25–27]. Exosomes could carry coding and non-coding RNA, single strand DNA, lipids, proteins and antigen-presenting molecules[28]. Exosomes are known for its function in antigen presentation and cell-cell communication. Consequently, they are recognized as important biomarkers of human disease[29]. The glaucoma-causing protein, myocilin, is an intracellular protein and released from TM cells by exosomes[18, 22, 30]. A previous study showed the physical properties of AH exosomes and pointed out that the exosomes in AH contain characteristic miRNAs[20].

In recently years, there is growing interest in studying the exosomal miRNAs for POAG. We still lack an understanding whether TGF- β 2 treatment TM cells, a model of glaucomatous TM dysfunction, can produce changes in exosomal cargo and function especially for exosomal miRNAs. The overall goal of this study was to identify and characterize exosomal miRNAs expression profiles in human trabecular meshwork cells (HTMCs) treatment by TGF- β 2 and speculate their potential target via bioinformatics analysis. The finding could be further developed as therapeutics for the POAG diagnosis and treatment.

Materials And Methods

Cell culture and treatment

Primary human trabecular meshwork cells (Cat. No. 6590, ScienCell, San Diego, CA, USA; see <http://www.sciencellonline.com> for details) were cultured in TM cell growth medium (TMCM, Cat. No. 6591, ScienCell), which contains basic medium (BM, ScienCell), 2% fetal bovine serum (FBS, Cat. No. 0010, ScienCell), 1% TM cell growth supplement (Cat. No. 6592, ScienCell) and 1% penicillin/streptomycin solution (P/S, Cat. No. 0503, ScienCell). All experiments used TM cells from passage 3 to 6 cultures. When the cells were 80 to 90% confluent, 5 ng/ml recombinant human TGF- β 2 (R&D Systems, Minneapolis, MN, USA) or 4 mM HCl solution containing 0.1% human serum albumin as vehicle was then added into 15 ml of media supplemented with 2% exosome-free FBS as described previously and the cells were incubated for 24 h[31]. TM cell isolation, characterization and culture were showed the previous study[32].

Conditioned medium collection and exosome isolation

To better understand the role of exosomes in HTM biology, our first step was to purify exosomes from conditioned media of primary HTMCs monolayers. After 24 h treatment, the cells culture supernatant was collected and exosomes were isolated by differential ultracentrifugation as previously described[33]. Briefly, the cells culture supernatant was centrifuged for 5 min at 300 g and then 10 min at 2000 g to eliminate cellular contamination. Then, the supernatant was centrifuged at 10000 g for 30 min and filtered by a 0.22 μ m filter (Millipore, USA), followed by ultracentrifugation at 100000 g for 1 h to pellet the exosomes. The pellets were washed in 20 ml cold PBS and pelleted again at 100000 g for 1 h, and resuspended in sterile PBS. All the operations were performed at 4 °C. The extracted exosomes were allocated and stored at -80 °C or used for the downstream experiments. The ultrastructure of exosomes was examined by transmission electron microscope (TEM) as previously described[33]. For the purpose of demonstrating the particle size distribution, nanoparticle tracking analysis (NTA) was performed using NanoSight NS300 (Malvern Instruments, Inc., Westborough, MA, USA) according to the operating instructions. Exosomal membranes were enriched in endosome-specific tetraspanins [34]. Then, we used Western Blot to detect the expression of the common exosomal markers CD63, CD81 and CD9 as previously described[35].

Total exosomal miRNAs extraction and analysis

To determine the expression changes of exosomal miRNAs derived from HTMCs when they were treated with TGF- β 2, we used miRNA gene chip to analysis. First, miRNAs were extracted from exosomes using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) as previously described[36]. A miRNA library preparation was conducted under the assistance of Shanghai Genechem Co., Ltd. The quality and concentration of miRNAs were assessed using NanoDrop 2000 Spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.). MiRNAs content were assessed using an Agilent 2200 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). Total miRNAs were labeled using FlashTag Biotin HSR labeling kits (Affymetrix, CA, USA) and hybridized to Affymetrix Gene Chip miRNA 4.0 Arrays, and scanned using an Affymetrix GCS3000 Gene Array Scanner according to manufacturer's protocol (Affymetrix, Santa Clara, CA, USA).

Bioinformatics analysis

The comprehensive function annotations of the potential targets of exosomal miRNAs were performed with Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis based on the DAVID 6.7 software (<http://david.abcc.ncifcrf.gov/home.jsp>). GO analysis was applied to predict the main functions of the target genes according to the GO project. Pathway analysis was performed to determine the significant pathway of the differential genes according to the KEGG. MiRNA-target gene network was established on the basis of GO and KEGG predicted data for the illustration of the relationship between miRNAs and their target genes. The target genes of exosomal miRNAs were predicted using StarBase (<http://starBase.sysu.edu.cn/>).

Statistical analysis

Fisher's exact test and χ^2 were used to classify the GO category and select the significant pathway. All GO terms and signaling pathways were analyzed with a threshold of significance that was defined by a *P*-value of < 0.05. Unless otherwise noted, all results were obtained through a minimum of three independent experimental replications.

Results

Human trabecular meshwork cell released exosomes

Like most other cells, cultured HTMCs can also release extracellular vesicles with properties consistent with exosomes[21]. After isolation, the morphology of exosome pellets was examined by TEM revealing membrane-bound particles that were homogeneous in appearance (Fig. 1A). In addition, the size distribution of exosome pellets was analyzed using NTA and the results revealed prospective diameters which ranged from 30–120 nm (Fig. 1B). Furthermore, we detected three well-established surface markers CD63, CD81 and CD9 for exosomes (Fig. 1C).

Exosomal miRNAs significantly altered when treated with TGF- β 2

The results of miRNA microarray analysis showed that 23 miRNAs up-regulated and 3 exosomal miRNAs down-regulated which had more than 2-fold compared to non-treated cells (Table 1). The top three up-regulated miRNAs were hsa-miR-6087 (4.45-fold), hsa-miR-663a (4.37-fold) and hsa-miR-6821-5p (3.18-fold). In addition to the 23 miRNAs, there were 3 down-regulated miRNAs: hsa-miR-6800-3p, hsa-miR-6716-3p and hsa-let-7i-5p. Using unsupervised hierarchical clustering analysis, a heat map was constructed based on differentially expressed miRNAs (Fig. 2). All of these data indicating that TGF- β 2 can affect miRNAs expression in exosomes from HTMCs supernatant.

Table 1
The change more than 2-fold for exosomal miRNAs of miRNA microarray analysis

Transcript ID	Alignments	Accession	Regulation	Fold Change
hsa-miR-6087	chrX:108297774–108297791 (+)	MIMAT0023712	up	4.454135148
hsa-miR-663a	chr20:26188879–26188900 (-)	MIMAT0003326	up	4.37625458
hsa-miR-6821-5p	chr22:50356514–50356536 (+)	MIMAT0027542	up	3.180286342
hsa-miR-1469	chr15:96876490–96876511 (+)	MIMAT0007347	up	2.71177347
hsa-miR-3665	chr13:78272210–78272227 (-)	MIMAT0018087	up	2.701236581
hsa-miR-2861	chr9:130548250–130548268 (+)	MIMAT0013802	up	2.637705202
hsa-miR-4466	chr6:157100844–157100861 (-)	MIMAT0018993	up	2.561762387
hsa-miR-1915-3p	chr10:21785505–21785524 (-)	MIMAT0007892	up	2.547317283
hsa-miR-6090	chr11:128392325–128392343 (+)	MIMAT0023715	up	2.54469603
hsa-miR-3656	chr11:118889702–118889718 (+)	MIMAT0018076	up	2.497602119
hsa-miR-6727-5p	chr1:1247919–1247941 (-)	MIMAT0027355	up	2.410969388
hsa-miR-8069	chr21:15096563–15096585 (+)	MIMAT0030996	up	2.32437042
hsa-miR-6125	chr12:62654199–62654218 (+)	MIMAT0024598	up	2.297376008
hsa-miR-3960	chr9:130548158–130548177 (+)	MIMAT0019337	up	2.28422801
hsa-miR-4508	chr15:23807254–23807270 (-)	MIMAT0019045	up	2.211112351
hsa-miR-6800-3p	chr19:50335333–50335353 (+)	MIMAT0027501	down	2.195221729
hsa-miR-6088	chr19:45939912–45939931 (+)	MIMAT0023713	up	2.189426977
hsa-miR-5787	chr3:50264870–50264889 (+)	MIMAT0023252	up	2.099719579
hsa-miR-4516	chr16:2183121–2183137 (+)	MIMAT0019053	up	2.086148223
hsa-miR-3940-5p	chr19:6416481–6416500 (-)	MIMAT0019229	up	2.075025885
hsa-miR-6716-3p	chr11:118514766–118514787 (+)	MIMAT0025845	down	2.074061011

Transcript ID	Alignments	Accession	Regulation	Fold Change
hsa-let-7i-5p	chr12:62997471–62997492 (+)	MIMAT0000415	down	2.069196993
hsa-miR-6816-5p	chr22:20102249–20102269 (-)	MIMAT0027532	up	2.060753578
hsa-miR-3196	chr20:61870140–61870157 (+)	MIMAT0015080	up	2.05297539
hsa-miR-6724-5p		MIMAT0025856	up	2.050079703
hsa-miR-6869-5p	chr20:1373584–1373605 (-)	MIMAT0027638	up	2.020174617

Exosomal miRNA-miRNA co-expression network

To gain a better understanding of how these miRNAs relate to one another, we then constructed the co-expression network of exosomal miRNAs (Fig. 3). In total, except hsa-miR-6724-5p, hsa-miR-6800-3p and hsa-miR-6716-3p, the other 23 miRNAs were included in the interaction network. In the interaction network, many up-regulated miRNAs have a positive correlation with another miRNA. Hsa-miR-1915-3p, hsa-miR-6727-5p and hsa-miR-3960 displayed the highest co-expression degree compared with other miRNAs. Has-let-7i-5p, which was down-regulated in exosome had a negative correlation with miR-4058. However, hsa-miR-6800-3p and hsa-miR-6716-3p weren't in this network. The co-expressed interaction network indicated that there may be synergistic and antagonistic effects between miRNAs. One single miRNA might interact with other several miRNAs and vice versa.

GO and KEGG pathway enrichment analysis

To interpret possible physiological or pathological processes and pathways regulated by the identified miRNAs, their putative target genes were subjected to GO annotation and KEGG pathway analysis (Fig. 4A, B). The results revealed that a total of 469 GO terms were involved in biological processes with $P < 0.05$ (The top 3: antigen processing and presentation, regulation of microtubule cytoskeleton organization and regulation of transcription from RNA polymerase II promoter in response to stress). Up to 102 GO terms were in relation to cellular components with $P < 0.05$ (The top 3: spliceosomal complex, endoplasmic reticulum lumen and neuron projection cytoplasm). There were 94 GO terms related to molecular function with $P < 0.05$ (The top 3: insulin-like growth factor I binding, nucleoside-triphosphatase regulator activity and transferase activity, transferring acyl groups). To provide information about the possible functions of 26 differentially expressed miRNAs, target prediction analysis was conducted by StarBase. The top 5 pathways, with the lowest P -value, were the p13k-akt signaling pathway, human papillomavirus infection, human cytomegalovirus infection, MAPK signaling pathways and proteoglycans in cancer. These results indicated that exosomal miRNAs involve a variety of cellular physiological and pathological processes.

Selective signaling pathways-exosomal miRNA co-expression network

Many signaling pathways are involved in the physiological and pathological changes of TM cells, and thus participate in the pathogenesis of POAG, like p53 signaling pathway[37, 38], focal adhesion[39, 40], MAPK signaling pathway[41, 42]. We then constructed the co-expression network of selective signaling pathways and exosomal miRNAs (Fig. 5). This network indicated that one single miRNA was bound up with a couple of signaling pathways and vice versa.

Discussion

Previous studies suggested that elevated level of TGF- β 2 in AH of POAG maybe central to the pathologic behavior of the disease[5, 7, 8]. However, until now, a comprehensive investigation of the effects of TGF- β 2 has not been presented. MicroRNAs housed within exosomes have emerged in recent years as critical modulators to synchronize AH production and drainage for ultimately IOP homeostasis[43] [20]. Our study was designed to test whether a famous model of glaucomatous TM dysfunction, TGF- β 2 treatment, can produce pathological changes in exosomal cargo and function. Furthermore, we want to reveal possible physiological or pathological processes and pathways involving pathogenesis of POAG regulated by the identified exosomal miRNAs.

According to the results of microarray analysis, 23 miRNAs were revealed to be up-regulated in the model group compared with the control group. Hsa-miR-6087, hsa-miR-663a and hsa-miR-6821-5p were the top three up-regulated exosomal miRNAs, whereas hsa-miR-6800-3p, hsa-miR-6716-3p, and hsa-let-7i-5p were revealed to be markedly down-regulated. Hsa-miR-6087 has been previously reported to act as an important mediators of cancer[44, 45], intermediate monocytes [46] and regulation of cell differentiation[47]. Has-miR-663a is located in the chromosome 20q11.1 and has been reported to be closely related with the biological behavior of cell differentiation, inflammation, autoimmune diseases, and cancer[48–50]. Let-7 was discovered early as a member of the large class of non-protein-coding RNAs. Dysregulated expression of the let-7i-5p is associated with various diseases such as cancer, and cardiovascular[51]. These exosomal miRNAs of AH may play a crucial role in the cell-cell communication for pathological processes of TM from POAG patients.

Previous study showed that significant up-regulation of mir-518d and mir-143, and significant down-regulation of mir-660 expression were observed in AH from POAG patients [52]. For this phenomenon, we infer the miRNAs in AH are not parallel to the miRNAs of the exosomes. To date, the specific source of miRNAs in AH is unknown. Variable intraocular cells can secrete exosomes into the AH, and numerous molecules, particularly miRNAs, have been shown to change in response to ocular disease or injury. Recent study shows that non-pigmented ciliary epithelium cell (NPCE)-derived exosomes could affect canonical Wnt signaling in TM cells, a pathway involved in IOP regulation[53]. Thus, we suspect that the changes of exosomal miRNAs mediate cell-cell communication and play a major role in the crosstalk between TM cells and the macro-/microenvironment.

The miRNA-miRNA interaction network showed that during the occurrence and development of diseases. Many miRNA molecules are involved in the process. The term interaction means one miRNA may interact and regulate the stability or expression of the other one or the others. Each miRNA regulates a broad spectrum of target genes and affects many signaling pathways. Therefore, disruption of a single miRNA expression may only limit an inhibitory effect, whereas joint interference of multiple miRNAs may be effective. Thus, determining how to silence multiple abnormally expressed miRNAs simultaneously in order to enhance the efficacy of disease treatments is important. For the reason, we used DIANA-TarBase to predict the potential targets of 26 exosomal miRNAs. Unlike miRDB and TargetScan, DIANA-TarBase was the first database aiming to catalog published experimentally validated interactions between miRNAs and genes. The GO analysis results revealed that the exosomal miRNAs were involved in biological processes, cellular components and molecular functions, including antigen processing and presentation, spliceosomal complex and insulin-like growth factor I binding. These miRNAs are not specific to a single gene. These miRNAs alter the expression of a number of genes instead of a single gene, and these genes may be involved in a single cellular process like extracellular matrix remodeling[54].

KEGG pathway enrichment analysis also revealed that the p13k-akt signaling pathway and MAPK signaling pathways may be involved in the process. We then constructed the co-expression network of selective signaling pathways and exosomal miRNAs. Overall, these studies indicate that exosomes and their cargoes can be used as biomarkers for glaucoma, potentially serving as an early diagnostic marker, and may also contribute directly to the progression of the disease. TGF- β 2 cause not only gene and protein expression changes in the HTM cells, but also can alter exosomal miRNAs expression. These miRNAs enlisted in the current study represent only a small proportion of genes in exosomes. This condition may be correlated with the specific condition of *in vitro* culture of the cells.

Conclusions

The present study provided new information regarding the potential role of miRNAs in exosomes. MicroRNAs in AH exosome can be stably stored under different conditions, indicating that exosomal miRNAs are potential biomarkers or novel therapeutic tools of POAG.

Abbreviations

POAG:primary open-angle glaucoma; TM:trabecular meshwork; AH:aqueous humor; ECM:extracellular matrix; HTMCs:human trabecular meshwork cells; TEM:transmission electron microscope; NTA:nanoparticle tracking analysis; GO:Gene Ontology; KEGG:Kyoto Encyclopedia of Genes and Genomes

Declarations

Ethics approval and consent to participate

Not applicable

Consent to publish

Not applicable

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests

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Authors' Contributions

JLZ and JWL performed the experiments. YW and HJG conceived the research, and critically reviewed the manuscript and interpreted the data. All authors read and approved the final manuscript.

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Figures

Figure 1

The exosomes were released from human trabecular meshwork cell (HTMCs). (A) Representative electron microscopy image of exosomes from HTMCs. (B) Nanoparticle tracking analysis detected the size distribution of exosomes isolated from HTMCs. (C) Western blot analysis of exosomal markers. The color Key represent Z-score(A z-score less than 0 represents an element less than the mean, greater than 0 represents an element greater than the mean and equal to 0 represents an element equal to the mean.).

Figure 2

The different miRNAs expression profiles obtained from the comparison of exosomal miRNAs isolated from HTMCs supernatant. The heat map revealed the distinct miRNA expression profiles between control and TGF- β 2 treated group. The inclusion criteria was a 2-fold difference of log₂ (fold-change) in either direction with a P-value <0.05. Red signal, high relative expression; green signal, low relative expression.

Figure 3

Exosomal miRNA-miRNA co-expression network. The network revealed the interactions among exosomal miRNAs. Upregulated miRNAs are marked red. Downregulated miRNAs are marked green.

A

B

Figure 4

Bioinformatics analysis of exosomal miRNAs. (A) GO annotation of predicted targets. (B) KEGG pathway enrichment analysis of predicted targets. GO terms and KEGG pathway both with a P-value <0.05 were selected.

Figure 5

Selective signaling pathways-exosomal miRNA co-expression network. The network revealed the interactions between exosomal miRNAs and genes signaling pathways. Upregulated miRNAs are marked red. Downregulated miRNAs are marked green.