

Expression profiling of microRNAs in optineurin (E50K) mutant transgenic mice

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Abstract. An E50K substitution in the transcription factor optineurin (*OPTN*) induces primary open-angle glaucoma (POAG). To explore the potential role of microRNAs (miRNAs) in E50K *OPTN*-induced POAG, miRNA expression profiling was performed on retinal samples from *OPTN* (E50K) transgenic and wild-type mice. The retinas were collected from 30 transgenic and 30 wild-type mice, and miRNA expression was evaluated using a genome-wide miRNA microarray. miRNAs that were differentially expressed in retinal samples from *OPTN* (E50K) transgenic mice were identified and validated by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Additional gene ontology and signaling pathway analyses were performed using bioinformatics tools. A total of 48 miRNAs exhibited increased or decreased expression in the retinas from *OPTN* (E50K) transgenic mice when compared with the expression in the retinas from wild-type mice. A total of 5 miRNAs with increased expression in *OPTN* (E50K) transgenic mice could be grouped into one cluster as they belong to the miR-8 family and may act as regulators in the development of POAG in *OPTN* (E50K) transgenic mice. RT-qPCR results confirmed significantly increased expression of miR-141 in the retinas of *OPTN* (E50K) transgenic mice as compared to wild-type mice. In conclusion, these results show that certain miRNAs are differentially expressed in the retinas of *OPTN* (E50K) transgenic mice and may play roles in the pathogenesis of POAG induced by *OPTN* (E50K).

Introduction

Glaucoma causes irreversible blindness and is characterized by optic nerve degeneration and visual field defects (1). Primary

open-angle glaucoma (POAG) is the major form of glaucoma. An epidemiological study in Japan demonstrated that a large number of POAG cases were diagnosed as normal tension glaucoma (NTG) (2). Mutations in the optineurin (*OPTN*) gene that encodes amino acid substitutions, such as E50K, H486R and R545Q, have been associated with POAG and NTG (3-6). The substitution of glutamic acid by lysine at amino acid 50 (E50K) is exclusively associated with familial and sporadic forms of NTG (7). Previous studies have shown that E50K *OPTN* induces the apoptosis of retinal ganglion cells in transgenic mice models and progressive retinal degeneration exclusively in the peripheral region of the retinas, although the exact mechanism remains unclear (8).

MicroRNAs (miRNAs) bind to target messenger RNAs (mRNAs) and subsequently suppress protein expression via mRNA degradation or translational inhibition (9). Several reports have described the expression of miRNAs in the eye and specific miRNAs have been indicated in the pathogenesis of glaucoma. In one report, miRNA-24 was shown to regulate transforming growth factor β 1 processing during cyclic mechanical stress in human trabecular network cells (10). It is unclear whether miRNAs contribute to pathogenesis in *OPTN* (E50K) transgenic mice. To address whether additional miRNAs could play a role in *OPTN* (E50K)-induced POAG pathogenesis, miRNA profiling was performed of the retinal samples from *OPTN* (E50K) transgenic mice and the differentially expressed miRNAs were identified. Differential expression of miRNAs was validated by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The identification of the differentially expressed miRNAs due to mutant *OPTN* (E50K) expression is likely to provide a foundation for studying the molecular pathways contributing to POAG.

Materials and methods

Animals and sample collection. All the animal experiments in the study were performed according to the tenets of the National Institutes of Health Guidelines and Regulations on the Care and Use of Animals in Research. Transgenic mice engineered to express the E50K mutant human *OPTN* in the retina were as described previously (11). Retinas from 8-month-old transgenic and wild-type mice were collected for genome-wide miRNA expression analysis. Retinas from 3 groups of 10 transgenic mice (E1, E2 and E3) and 3 groups of

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10 wild-type mice (W1, W2 and W3) were pooled for analysis and independently analyzed for miRNA expression. Retinal samples were frozen at -80°C until analysis.

miRNA microarray study. miRNA microarray experiments were performed at CapitalBio Corp. (Beijing, China). Total RNA was extracted with TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) from retinal samples collected from transgenic and wild-type mice. Samples were labeled and hybridized to a GeneChip[®] miRNA Array (V3.0; Affymetrix, Santa Clara, CA, USA). Raw data were normalized and analyzed using the miRNA QC Tool software (Affymetrix). The analysis focused on miRNAs with a ≥ 1.5 -fold expression difference in retinal samples from *OPTN* (E50K) transgenic mice as compared to wild-type control retinal samples.

miRNA target gene prediction and function analysis. Prediction of miRNA target genes was performed using a computational approach. The prediction of miRNA target genes was performed using 3 different miRNA target prediction algorithms: PicTar, miRanda v5 and TargetScan v6.0. For each algorithm, the potential binding sites in the mRNA 3'-untranslated region (3'-UTR) of target genes were identified according to specific base-pairing rules.

RT-qPCR miRNA analysis. To confirm the results from the miRNA microarray, RT-qPCR analysis of mmu-miR-141 was performed on the same total RNA used for microarray analysis. First-strand cDNA synthesis was performed from equal amounts of total RNA using an All-in-One[™] miRNA First-Strand cDNA Synthesis kit (GeneCopoeia, Guangdong, China) according to the manufacturer's instructions. Primers were designed and synthesized by GeneCopoeia.

RT-qPCR was performed using the All-in-One[™] qPCR Mix on LightCycler 480 system (Roche, Basel, Switzerland). The levels of an endogenous control, U6, were used to normalize the expression levels of each miRNA. All the reactions were performed in triplicate and included controls. The fold-change in miRNA expression was calculated using the comparative Ct method. Data are presented as the fold-change relative to expression in the retinal samples of wild-type mice.

Statistical analysis. All the results are expressed as the mean \pm standard deviation. Statistical analysis was performed with the Student's t-test to identify statistically significant differences using commercial software (SigmaPlot; Systat Software Inc., San Jose, CA, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Global miRNA profiling in *OPTN* (E50K) transgenic and wild-type mice. Retinal miRNA expression patterns were evaluated using pooled retinal samples for 3 separate groups of 10 transgenic mice (E1, E2 and E3) and 10 wild-type mice (W1, W2 and W3). For each pooled sample, $> 2 \mu\text{g}$ of retinal RNA was harvested. Spectrophotometric analysis of RNA purity was performed by measuring absorbance at 260 and

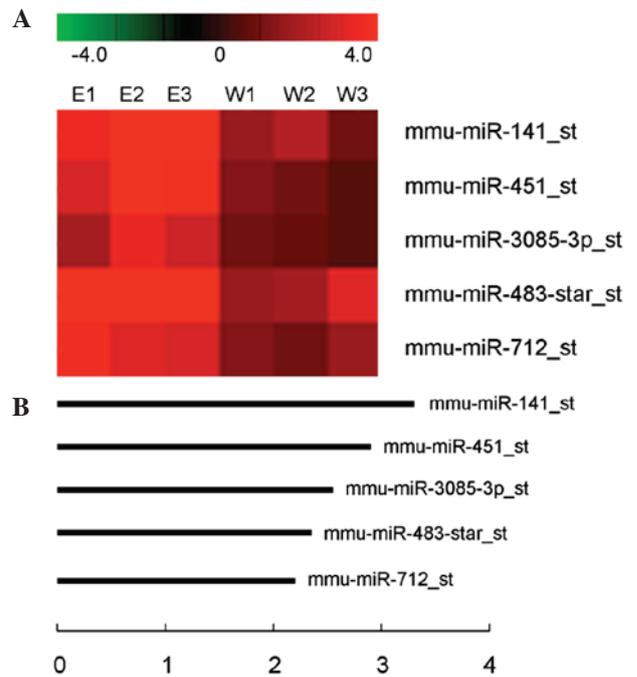


Figure 1. MicroRNA (miRNA) profiles were performed on the retinas of optineurin (*OPTN*) (E50K) transgenic and wild-type mice. (A) Hierarchical clustering was performed with normalized miRNA data (fold-change ≥ 1.5) that passed the Student's t-test ($P < 0.05$). A total of 5 miRNAs were identified based on significant expression differences in the retinas of *OPTN* (E50K) mutation transgenic mice as compared to wild-type mice. E1-E3 and W1-W3 represent miRNA expression data for three independent samples of pooled retinal RNA from *OPTN* (E50K) transgenic or wild-type mice, respectively. For each miRNA, the intensity of the red color reflects the expression levels. (B) Fold-change in miRNA expression in the retinas of *OPTN* (E50K) transgenic mice as compared to wild-type mice, determined by miRNA array.

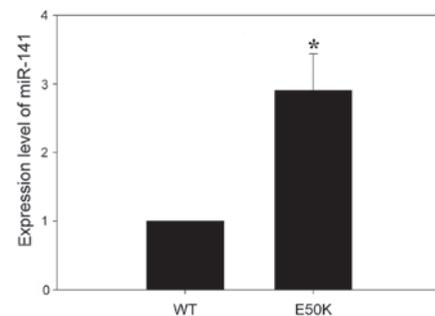


Figure 2. Validation of selected microarray data by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Relative expression levels of miR-141 were determined by RT-qPCR. mmu-miR-141 expression was markedly upregulated in the retinas of optineurin (E50K) transgenic mice compared to wild-type (WT) mice. Values are means \pm standard deviation and are expressed relative to internal control (U6; $n = 3$ for each group; $*P < 0.05$, Student's t-test).

280 nm. All samples exhibited 260/280 nm absorbance ratios > 1.96 , indicating isolation of high-quality retinal RNA.

Global miRNA expression patterns were determined for each sample by microarray analysis. The analysis focused on specific miRNAs exhibiting differential expression levels in the retinas from *OPTN* (E50K) transgenic mice as compared to wild-type mice by identifying miRNAs with a ≥ 1.5 -fold-change in expression level and $P \leq 0.05$. Based on this threshold, 48 miRNAs were differentially expressed in *OPTN*

Table I. MicroRNA (miRNA) profiles were performed on the retinas of optineurin (E50K) transgenic and wild-type mice. miRNAs are ranked in order of fold-change, from the largest to the smallest.

miRNA ID	Fold-change	miRNA sequence
mmu-miR-141	3.2023	UAACACUGUCUGGUAAGAUGG
mmu-miR-451	2.8473	AAACCGUUACCAUACUGAGUU
mmu-miR-200a	2.6426	UAACACUGUCUGGUAACGAUGU
mmu-miR-483*	2.5317	UCACUCCUCCCCUCCCGUCUU
mmu-miR-3057-3p	2.4620	UCCACAGGCCAGCUCAUAGC
mmu-miR-877*	2.4260	UGUCCUCUUCUCCUCCUCCCA
mmu-miR-3102-3p.2	2.4235	CUCUACUCCUGCCCCAGCCA
mmu-miR-429	2.3851	UAAUACUGUCUGGUAUGCCGU
mmu-miR-3085-3p	2.3604	UCUGGCUGCUAUGGCCCCUC
mmu-miR-712	2.3241	CUCCUUCACCCGGGCGGUACC
mmu-miR-223	2.2929	UGUCAGUUUGUCAAUACCCCA
mmu-miR-211	2.2731	UCCCCUUGUCAUCCUUGCCU
mmu-miR-31*	2.2721	UGCUAUGCCAACAUAUUGCCAUC
mmu-miR-27a	2.0945	UUCACAGUGGCUAAGUCCGC
mmu-miR-3963	2.0212	UGUAUCCACUUCUGACAC
mmu-miR-1a	2.0141	UGGAAUGUAAAGAAGUAUGUAU
mmu-miR-3968	1.9957	CGAAUCCACUCCAGACACCA
mmu-miR-200b	1.9956	UAAUACUGCCUGGUAUGAUGA
mmu-miR-200a*	1.9578	CAUCUUACCGGACAGUGCUGGA
mmu-let-7b*	1.9529	CUAUAACAACCUACUGCCUCCCC
mmu-miR-3092	1.8662	GAAUGGGGCGUUUCCCCUCC
mmu-miR-200b*	1.8444	CAUCUUACUGGGCAGCAUUGGA
mmu-miR-21	1.8270	UAGCUUAUCAGACUGAUGUUGA
mmu-miR-205	1.7966	UCCUUCAUUCCACCGGAGUCUG
mmu-miR-203	1.7911	GUGAAAUGUUUAGGACCACUAG
mmu-miR-3072	1.7820	UGCCCCUCCAGGAAGCCUUCU
mmu-miR-27a*	1.7546	AGGGCUUAGCUGCUUGUGAGCA
mmu-miR-30e	1.7120	UGUAAACAUCUUGACUGGAAG
mmu-miR-28c	1.6958	AGGAGCUCACAGUCUAUUGA
mmu-miR-1843b-5p	1.6886	AUGGAGGUCUCUGUCUGACUU
mmu-miR-122	1.6574	UGGAGUGUGACAAUGGUGUUUG
mmu-miR-467d*	1.6427	AUAUACAUAACACACCCUACAC
mmu-miR-34c*	1.6416	AAUCACUAACCACACAGCCAGG
mmu-miR-148a	1.6200	UCAGUGCACUACAGAACUUUGU
mmu-miR-1298*	1.5997	CAUCUGGGCAACUGAUUGAACU
mmu-miR-1949	1.5995	CUAUACCAGGAUGUCAGCAUAGUU
mmu-miR-15a	1.5988	UAGCAGCACAAUUGGUUUUGUG
mmu-miR-497	1.5903	CAGCAGCACACUUGGUUUUGUA
mmu-miR-5127	1.5743	UCUCCCAACCCUUUCCCCA
mmu-miR-200c	1.5620	UAAUACUGCCGGGUAUGAUGGA
mmu-miR-467b*	1.5491	AUAUACAUAACACACCAACAC
mmu-miR-205*	1.5424	GAUUUCAGUGGAGUGAAGCUCA
mmu-miR-744*	1.5254	CUGUUGCCACUAACCUCAACCU
mmu-miR-140	1.5219	CAGUGGUUUUACCCUAUGGUAG
mmu-miR-143	1.5215	UGAGAUGAAGCACUGUAGCUC
mmu-miR-19b	1.5059	UGUGCAAUCCAUGCAAAACUGA
mmu-miR-106b	1.5015	UAAAGUGCUGACAGUGCAGAU
mmu-miR-335-5p	1.5000	UCAAGAGCAAUACGAAAAAUGU

(E50K) transgenic retinal samples as compared to wild-type retinal samples, representing 10% of the 48 miRNAs represented on the array (Table I). Of these, 5 miRNAs passed the Volcano plot filtering screen to identify miRNAs that were expressed at significantly different levels in transgenic versus

wild-type retinas ($P < 0.05$; false discovery rate, < 0.05 ; Fig. 1A). Each of these miRNAs was upregulated 2.32-3.20-fold in the retinas of *OPTN* (E50K) transgenic mice compared to wild-type mice (Fig. 1B). *mmu-miR-141* was focused on as it exhibited the largest increase in expression in retinal samples

from transgenic mice and RT-qPCR was used to validate the miRNA microarray results. Consistent with the microarray results, RT-qPCR analysis demonstrated that mmu-miR-141 was expressed at significantly higher levels in the retinas of transgenic mice compared to wild-type mice (Fig. 2).

Computational prediction of potential target genes and network analysis. Candidate target genes for mmu-miR-141 were identified using three commonly used prediction algorithms to reduce the unpredictable number of false positives: PicTar, miRanda and TargetScan. The results of the miRNA-mRNA regulatory network analysis indicated that mmu-miR-141 belonged to the miR-8 family. The miR-8 family also includes mmu-miR-200a, mmu-miR-200b, mmu-miR-200c and mmu-miR-429, all of which were upregulated in the retinas of *OPTN* (E50K) transgenic mice by microarray but did not achieve statistical significance.

Discussion

The *OPTN* E50K mutation is the only mutation currently confirmed to play a causative role in NTG pathogenesis (2). Transgenic mice that overexpress *OPTN* E50K provide a model system for elucidating the molecular mechanisms leading to POAG (8). While the majority of previous studies have focused on direct protein-protein interactions, differences in the expression levels of core proteins have also been noted and may contribute to POAG pathogenesis. The mechanisms accounting for differential expression of proteins indicated in POAG are not clear.

miRNAs can regulate gene expression through sequence complementarity to the 3'-UTRs of target mRNAs. miRNA binding to target mRNAs can result in translational repression through mRNA degradation or translational inhibition (12). Accumulating evidence suggests that miRNAs act as novel cellular senescence regulators. However, there is little information regarding the potential involvement of miRNAs in the mediating effects originating from the expression of mutant proteins associated with disease processes, such as *OPTN* (E50K). The present results suggest that the miRNAs miR-141, miR-200a, miR-200b, miR-200c and miR-429, which all belong to the miR-8 family, may be critical regulators of POAG induced by the *OPTN* (E50K) mutation.

The miR-8 family has been demonstrated to regulate numerous biological processes, including the response to osmotic stress in zebrafish embryos (13) and the response to steroid signaling in *Drosophila* (14). miR-200b, miR-200c and miR-429 are *TBK1* gene-related miRNAs according to the miRbase database. The E50K mutation in *OPTN* may enhance its interaction with *TBK1* and affect *OPTN* function by rendering *OPTN* insoluble (15). However, no experiments have been performed to fully elucidate the mechanisms by which the *OPTN* (E50K) mutation induces POAG to date. Therefore, further research to understand the interaction of the *OPTN* (E50K) mutation with *TBK1* could improve the management of POAG.

In conclusion, the present study identified a number of differentially expressed miRNAs in the retinas of *OPTN* (E50K) mutant transgenic mice. The results suggest

several noteworthy directions for future research aimed at elucidating the role of miRNAs in glaucoma pathogenesis. Detailed analyses of miRNA expression patterns and the impact on retinal cell function may contribute to our understanding of the pathophysiological processes leading to POAG.

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