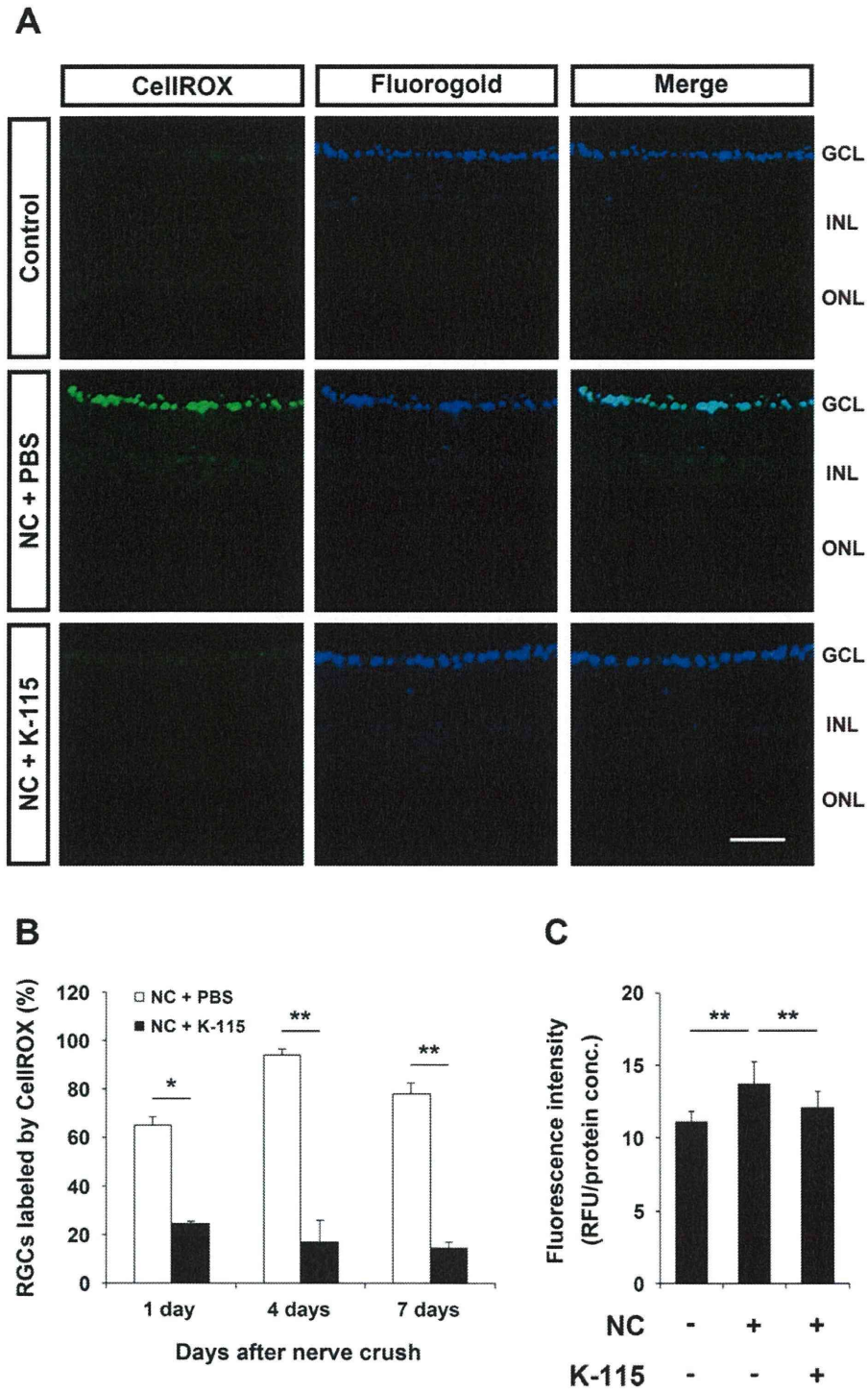

FIGURE 5. K-115 suppressed the time-dependent production of ROS in RGCs. (**A**) Representative fluorescence images of frozen sections confirming ROS production (*green*) in FG-labeled RGCs (*blue*) on day 4 after NC. K-115 treatment attenuated the NC-induced increase in CellROX fluorescence in RGCs. *Scale bar*: 100 μm. (**B**) Quantification of the number of CellROX positive cells among FG-labeled RGCs on days 1, 4, and 7 after NC injury. K-115 significantly reduced the number of CellROX-labeled cells among the retrogradely labeled RGCs at each time point ($^{*}P < 0.05$, $^{**}P < 0.01$; *error bars*, SD; PBS group: day 1 $n = 4$, days 4 and 7 $n = 6$; K-115 group: day 1, 4 $n = 6$, day 7 $n = 5$). (**C**) Measurement of ROS levels using a fluorophotometer. Fluorescence intensity (RFU) was normalized to protein concentration (mg/mL), which was determined with a BCA protein assay kit ($^{*}P < 0.05$, $^{**}P < 0.01$; *error bars*, SD; $n = 6$ in each group).

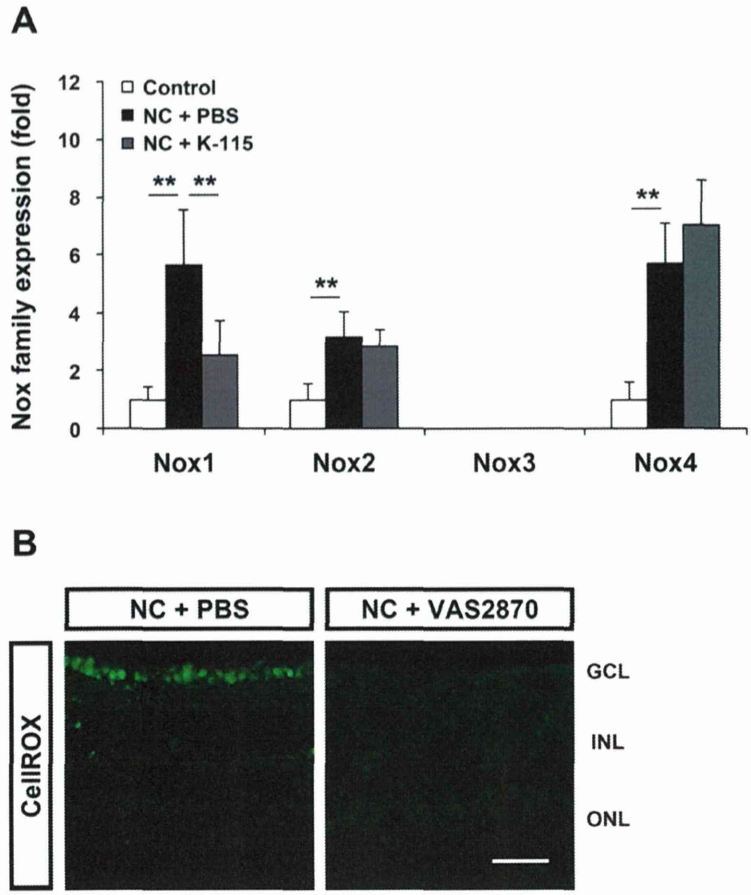

FIGURE 6. Involvement of the Nox family in NC-induced ROS production. (**A**) Treatment with K-115 suppressed induction of the Nox family, *Nox1-4* mRNA, at day 4 after NC. *GAPDH* was used as an internal standard (**$P < 0.01$; *error bars*, SD; $n = 8$ in each group). (**B**) Representative fluorescence images of frozen sections showing that axonal injury-induced ROS production was greatly reduced by treatment with the Nox inhibitor VAS2870 (10 pmol/eye) at day 4. VAS2870 was intravitreally injected within 5 minutes of NC ($n = 4$ in each group). *Scale bar*: 100 μm.

increased in the RGC layer, reaching a maximum on the fourth day after injury.[59]

Moreover, the increase in ROCK activity was not observed in other retinal layers, implying that this response to axonal lesions is specific to RGCs. However, we had difficulty at first finding evidence directly indicating which oxidative stress pathway was suppressed by the ROCK inhibitor K-115. Figure 3 shows our assessment of the ability of ROCK inhibitors to directly suppress oxidative stress. The antioxidant effect of K-115 was significantly lower than that of BHT. This suggests that K-115 did not act as an antioxidant reagent in this system, and led us to believe that ROCK inhibitors increase antioxidant activity through another mechanism. Looking to test this hypothesis, we assessed the gene expression pattern after NC of the Nox family, including *Nox1*, *2*, *3*, and *4*, with K-115 treatment. Previously, it was reported that the Nox family is expressed downstream of ROCK activity,[22,81,82,96] and indeed, we did find that the expression of *Nox1*, *2*, and *4* increased after NC, particularly *Nox1* and *2* in the RGCs. Previous findings that Nox1, 2, and 4 were expressed in surviving RGCs, and that RGCs had a significantly higher level of Nox1 than other members of the Nox family,[96] support our results. It has also been reported that *Nox2* is particularly expressed in the microglial cells.[97,98] The final results of the experiments reported here showed that only *Nox1* was suppressed after NC with K-115 treatment. This decrease in *Nox1* expression after K-115 treatment supported our hypoth-

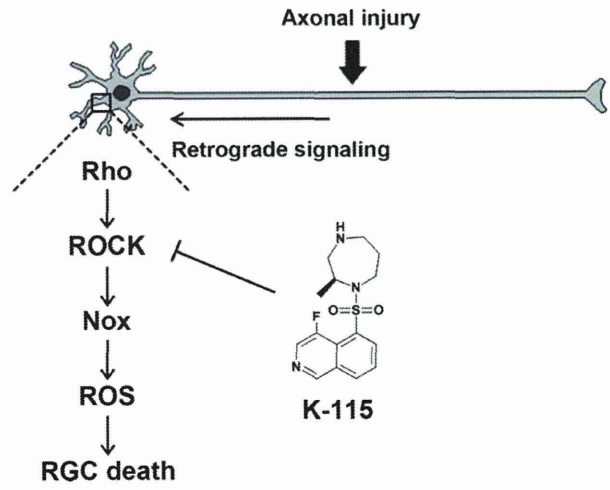

FIGURE 7. K-115 inhibited oxidative stress via the Rho/ROCK pathway in RGCs. Axonal injury resulted in Nox-mediated ROS production via the Rho/ROCK pathway, which was attenuated by K-115.

esis. Indeed, Nox1 was found to play a critical role in ischemia-induced oxidative stress and RGC death in experiments using the Nox inhibitor VAS2870.[96] Furthermore, since K-115 also significantly inhibits RGC death in ischemia-reperfusion models (Mizuno K, et al. *IOVS* 2007;48:ARVO E-Abstract 4805), this protective effect might have a similar mechanism that modulates Nox family expression.

It is widely accepted that mitochondrial ROS cause oxidative damage to nuclear DNA. Mitochondrial-derived death signaling has previously been reported to be an important pathway for RGC death induced by axonal damage.[99,100] Mitochondria are also known to be abundant in the optic nerve.[101] Previously, we detected ROS in the mitochondria of RGCs, suggesting that axonal damage affects mitochondrial function, which in turn triggers RGC death.[72] In the present study, we have confirmed that oxidative stress is also involved in NC-induced RGC death. In this study we present evidence, using CellROX staining, that NC-induced apoptosis in RGCs produced high amounts of ROS. Indeed, the reduction in the number of ROS-producing cells with K-115 treatment was confirmed by both counting the cells (Fig. 5B) and by a fluorophotometric analysis (Fig. 5C). However, the difference between the PBS and K-115 treatment groups, shown in Figure 5C, were smaller than in the groups shown in Figure 5B. The different results obtained from these two analyses raise the possibility that our fluorophotometric evaluation of the suppressive effect of K-115 on ROS production after NC may have been affected by the difficulty of measuring the fluorescence intensity of retinal lysates. ROS are generated by the process of isolating the retina itself, even if the retinas are immediately dissected in ice-cold DPBS. Therefore, we consider that counting the number of cells was the most suitable method to evaluate K-115's effect on ROS production. The metabolic processes involving the mitochondrial electron transport chain are known to contribute to the formation of harmful ROS. Furthermore, our results indicated that K-115 significantly suppressed NC-induced oxidative stress by inhibiting ROS production in RGCs.

Our results thus strongly suggest that the prevention of oxidative stress in the mitochondria or nucleus should be regarded as candidates for the treatment of glaucoma. Furthermore, we believe that we have shown that suppression of Rho activity also has the potential to be a new neuroprotective treatment for glaucoma, particularly NTG (the main type of glaucoma in Asian countries).[102]

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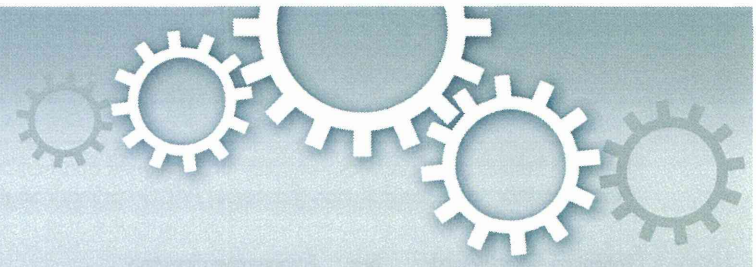

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Profiles of Extracellular miRNAs in the Aqueous Humor of Glaucoma Patients Assessed with a Microarray System

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Aqueous humor (AH) is one of the body fluids in the eye, which is known to be related with various ocular diseases, but the complete RNAs characteristic of the AH in patients is not yet known. The aim of this study was, with a microarray analysis, to reveal the disease-related extracellular miRNAs profiles in individual patients AH. 100 μl of AH was collected by anterior chamber paracentesis from 10 glaucoma, 5 cataract, and 5 epiretinal membrane patients. The extracted total RNAs were shorter than 200 nt, and their amount was 5.27 ± 0.41 ng in average. Among 530.5 ± 44.6 miRNA types detected in each sample with a microarray detectable 2019 types of matured miRNAs, 172 miRNAs were detected in all 10 glaucoma or control patients. From the glaucoma group, 11 significantly up-regulated and 18 significantly down-regulated miRNAs ($P < 0.05$ for both) were found to have areas under the curve better than 0.74 in a receiver operating characteristic analysis. They also formed a cluster composed only of glaucoma patients in a hierarchal cluster analysis. AH had a possibility of becoming a source of miRNA that can serve as a biomarker and a therapeutic target.

Increasing the quality of care expected by patients requires the development of improved diagnostic, prognostic and therapeutic monitoring tools for disease. Biomarkers of disease, including extracellular microRNAs (miRNAs) in bodily fluids, may play a role in achieving this purpose, as well as elucidating pathogenic mechanisms. MiRNAs have recently attracted attention for the prospect of earlier diagnosis and more precise application of existing therapies[1].

MiRNAs are small, non-coding RNA molecules, consisting of 19–22 nucleotides. They have been intensely studied since the discovery two decades ago of the role of the *lin-4* gene in regulating protein abundance[2,3] with the general goals of understanding their role in translational repression and messenger RNA cleavage in various biological processes[4–6], and elucidating pathologies in these processes[6–9]. After the recent discovery of circulating extracellular miRNAs in bodily fluids (e.g. plasma and serum), new searches for disease-related extracellular miRNAs have started in various fields[4,10–13].

There are three sources of extracellular miRNAs in the eye: tears, the vitreous humor, and the aqueous humor (AH)[11,14,15]. Studies of ocular fluids and other cellular tissues that used a proteomic approach have identified several disease-related proteins[16–19] including multiple Alzheimer's disease related peptides in the AH of eyes with glaucoma[20,21]. In comparison, research into ocular extracellular miRNAs is only at an early stage[11,14,15] and still has great potential to uncover novel, accurate and valuable biomarkers and therapeutic targets.

Glaucoma is one of the leading causes of blindness worldwide[22–24]. The axons of retinal ganglion cells, which extend to the brain through the optic nerve, are irreversibly damaged in glaucoma, but with early detection and proper medical treatment, it is possible to slow the progress of this damage. The present study focused on finding AH miRNAs related to glaucoma. The AH is the most frequently sampled ocular fluid in examinations and clinical studies of ocular disease. It is secreted by the ciliary epithelium, which supplies nutrients and removes metabolic waste from the avascular tissues of the eye such as the lens and cornea. The AH is excreted through the angle of the anterior chamber, which consists of the trabecular meshwork, uvea, and sclera. Pressure regulation and dysregulation in this system is associated with glaucoma[25]. Luna *et al* reported that miR-29b has a potential

Table 1 | Patient characteristics, summary of RNA yield and number of miRNAs detected by microarray. POAG : primary open angle glaucoma PEX : pseudoexfoliation glaucoma, PACG : primary angle closure glaucoma

Patient no.	Age (years)	Sex	Preoperative diagnosis	Note	Amount of total RNA (ng)	Number of detected genes
Cont. 1	71	M	Cataract	Hypertension	6.54	539
Cont. 2	77	M	Cataract	Dislipidemia, diabetic melllitus	4.59	821
Cont. 3	66	F	Cataract	Hypertension, dislipidemia	1.43	626
Cont. 4	66	F	Cataract		7.33	518
Cont. 5	69	F	Cataract	Hypertension, dislipidemia,	6.64	432
Cont. 6	69	F	Epiretinal Membrane	Hypertension	5.81	490
Cont. 7	84	F	Epiretinal Membrane	Hypertension, dislipidemia, diabetic melllitus	8.47	298
Cont. 8	80	M	Epiretinal Membrane	Hypertension	7.24	465
Cont. 9	74	F	Epiretinal Membrane	Hypertension	7.24	395
Cont. 10	55	F	Epiretinal Membrane	Dislipidemia	6.62	1026
Gla. 1	83	F	Glaucoma (POAG), Cataract	Hypertension, diabetic melllitus	6.06	602
Gla. 2	64	M	Glaucoma (PACG), Cataract		5.56	801
Gla. 3	87	M	Glaucoma (POAG), Cataract	Hypertension, dislipidemia	6.24	583
Gla. 4	75	F	Glaucoma (POAG)	Hypertension, dislipidemia	6.64	455
Gla. 5	58	F	Glaucoma (PEX)		7.21	519
Gla. 6	70	M	Glaucoma (PEX)		3.40	345
Gla. 7	63	M	Glaucoma (POAG)	Hypertension	2.75	186
Gla. 8	70	M	Glaucoma (POAG)	Hypertension	7.42	740
Gla. 9	82	M	Glaucoma (POAG)	Diabetic melllitus	3.48	344
Gla. 10	76	F	Glaucoma (POAG)		3.96	425

role in glaucoma with an *in vitro* assay of the trabecular meshwork cells[26,27]. MiRNAs in the AH may have a key role in pathological conditions related to glaucoma, but the entire miRNA profile of the glaucomatous AH has not yet been determined.

The discovery of novel miRNAs has been accelerating with advances in analytical technologies[28], and the total number of known human mature miRNAs has reached 2019 (miRBase Version 19, released on Aug. 1, 2012). The possibility of finding new and more useful biomarkers thus continues to rise. In this study, we used a microarray system able to detect all 2019 known mature miRNAs to analyze individual AH samples from patients with glaucoma, cataracts, and epiretinal membrane (ERM), with the aim of revealing specific diseases, individual expression characteristics, and the number of detectible miRNA types in the AH.

Results

Total RNA extraction from AH of ocular patients. Table 1 and supplementary Table S1 shows patient characteristics, quantity of RNA yielded, and the number of miRNAs detected by the microarray system. There was no significant difference in age between the 10 glaucoma patients (72.8 ± 3.0 years old) and the 10 control patients (cataract and epiretinal membrane, 71.1 ± 2.6 years old), who participated in this study (Supplementary Figure. S2a online). Intra ocular pressure of glaucoma group was significantly higher than control group (Supplementary Figure. S2b online). We were able to obtain high quality purified total RNA with the bioanalyzer. There was a significant quantity of RNA shorter than 200 nt, and no ribosomal RNA longer than 200 nt (specifically, 18S and 28S rRNA were absent) (Figure 1a). From a 100 μl sample of AH, we obtained 5.27 ± 0.54 ng of purified total RNA from the glaucoma patients and 6.19 ± 0.62 ng from the 10 control patients (the average for both groups was 5.73 ± 0.41 ng). There was no significant difference in the quantity of purified total RNA obtained from the two groups (Figure 1b).

Microarray analysis of miRNAs in AH. Even with the small amount of purified total RNA and without any amplification steps, bright spots were present on the microarray (Figure 2a). An average of 530.5 ± 44.6 miRNA types were detected in the 20 samples, with no correlation to the quantity of purified total RNA (R^2 = 0.0197, Table 1, Supplementary Fig. S2 online), and no significant difference in the number of miRNAs detected in the glaucoma patients (500.0 ± 59.7) or control patients (561.0 ± 68.2) (Figure 2b).

Statistical analysis of microarray data of AH miRNAs. All of the values observed were normalized to the expression value of hsa-miR-3940-5p, which was detected in all the samples ($p = 0.985$, ratio of 1.005, Supplementary Table S2 online). 57 miRNAs showed a statistically significant difference ($p < 0.05$, $q < 0.2$) in expression levels in the control patients and glaucoma patients (Supplementary Figure S2 online). In this group, hsa-let-7b-3p (Supplementary Table S3 online), has-miR-4507, has-miR-3620-5p, has-miR-1587, and has-miR-4484 were most significantly different ($p < 0.01$).

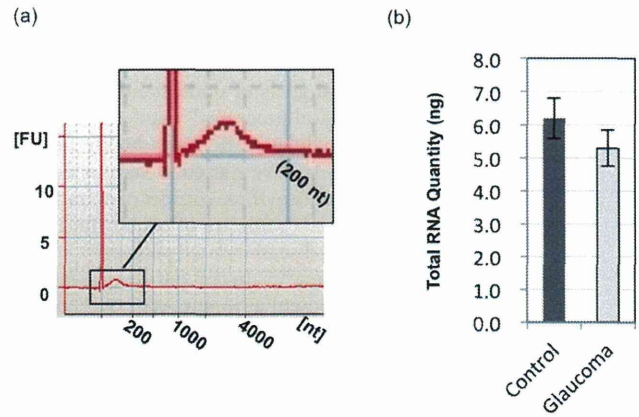


Figure 1 | (a) Total RNA validation, screens capture of Agilent 2100 Bioanalyzer electropherograms of representative human AH samples (total RNA), (b) comparison of total RNA quantity in glaucoma and control patients.

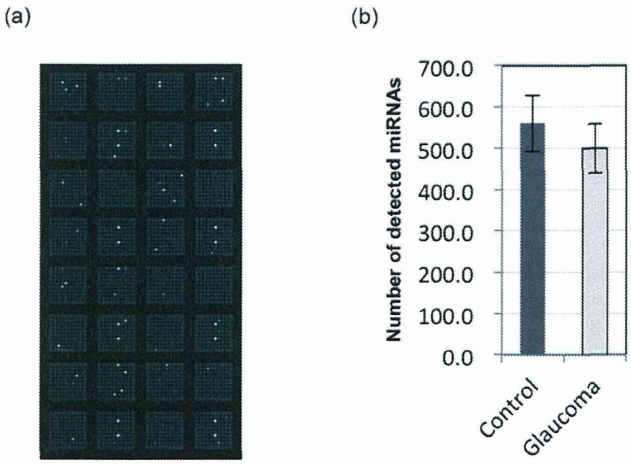
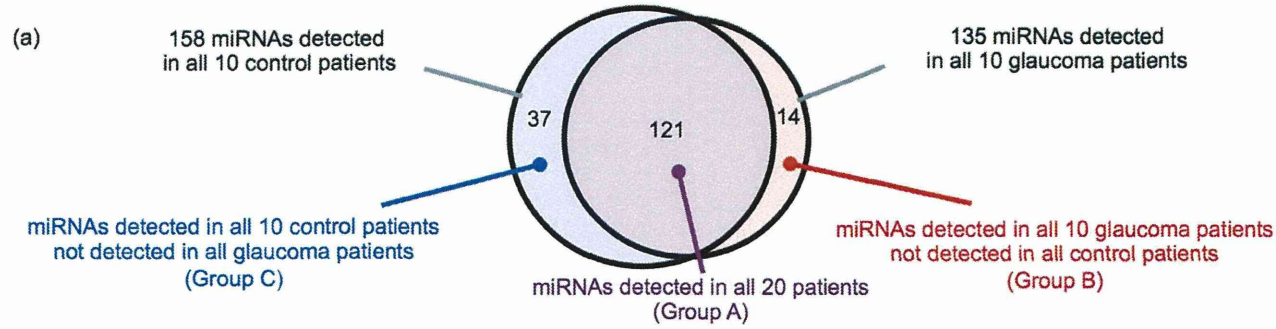


Figure 2 | (a) MiRNA microarray analysis, representative scanned image of a microarray capable of detecting 2019 miRNAs, and (b) comparison of detected miRNAs number in glaucoma and control patients.

The numbers of miRNA types detected in the all of 10 glaucoma patients and the all of 10 control patients were 158 and 135, respectively (Figure 3a). 121 of these miRNAs (group A) were detected in all 20 patients. 14 miRNAs (group B) were detected in all glaucoma patients, but not detected in any control patients. 37 miRNAs (group C) were detected in all control patients, but not detected in any glaucoma patients. Among the 121 miRNAs of group A, 18 miRNAs showed a statistically significant difference (<0.05) in expression level in the control patients and glaucoma patients, as shown in Fig. 3b and supplementary Table S4 online. Among these 18 miRNAs, 8 were up-regulated and 10 were down-regulated in the glaucoma patients. Among the 14 miRNAs of group B, 3 miRNAs were up-regulated in the glaucoma patients, a significant difference (<0.05) (Figure 3c, and Supplementary Table S5 online). Among the 37 miRNAs of group C, 8 miRNAs were down-regulated in the glaucoma patients, a significant difference (<0.05) dFigure 3c, and Supplementary Table S6 online). A receiver operating characteristic (ROC) analysis of the 11 up-regulated and 18 down-regulated miRNAs showed that they all had an area under the curve (AUC) greater than 0.74 (Figure 3b, c, and d). A hierarchal cluster analysis with these 29 miRNA markers showed a cluster composed only of glaucoma patients was formed (Figure 4).

Targets and pathway analysis of glaucoma specific AH miRNAs. DIANA-microT analysis was performed on 29 miRNA markers and hsa-let-7b-3p. Sixty-two and 266 sets of up- and down- regulated miRNAs/targets were predicted to have miTG scores > 0.97000 (Supplementary Table S7 and S8 online). Ten and 9 molecules, respectively, were targeted by more than one up- or down-regulated miRNA (Table 2). The top 2 molecular networks predicted by the IPA analysis were (1) RNA post-transcriptional modification, developmental disorders, hereditary disorders, and



(b) Group A

Name of miRNA	Average		p-value (t-test)	Fold change	Difference ((Glaucoma) -(Control))	AUC
	Control	Glaucoma				
hsa-miR-4507	0.095	0.069	0.005	0.728	-0.026	0.9
hsa-miR-3620-5p	0.211	0.131	0.006	0.622	-0.08	0.9
hsa-miR-4484	0.071	0.117	0.008	1.639	0.045	0.8
hsa-miR-5001-5p	0.23	0.166	0.011	0.718	-0.065	0.8
hsa-miR-6132	0.514	0.365	0.011	0.71	-0.149	0.8
hsa-miR-6515-3p	0.053	0.075	0.011	1.413	0.022	0.8
hsa-miR-4467	0.972	0.768	0.013	0.79	-0.204	0.8
hsa-miR-3663-3p	0.135	0.193	0.015	1.432	0.058	0.9
hsa-miR-187-5p	0.089	0.047	0.02	0.524	-0.042	0.8
hsa-miR-4433-3p	0.044	0.064	0.022	1.474	0.021	0.8
hsa-miR-6717-5p	0.207	0.395	0.027	1.909	0.188	0.9
hsa-miR-6722-3p	0.213	0.11	0.027	0.517	-0.103	0.8
hsa-miR-4725-3p	0.049	0.078	0.029	1.588	0.029	0.8
hsa-miR-1202	0.044	0.061	0.032	1.388	0.017	0.8
hsa-miR-3197	0.258	0.375	0.045	1.454	0.117	0.8
hsa-miR-4749-5p	0.159	0.126	0.046	0.794	-0.033	0.8
hsa-miR-1260b	0.683	0.45	0.046	0.659	-0.233	0.9
hsa-miR-4634	0.185	0.136	0.05	0.738	-0.048	0.8

(c) Group B

Name of miRNA	Average		p-value (t-test)	Fold change	Difference ((Glaucoma) -(Control))	AUC
	Control	Glaucoma				
hsa-miR-4259	0.033	0.065	0.015	1.981	0.032	0.8
hsa-miR-92a-2-5p	0.023	0.053	0.022	2.331	0.030	0.8
hsa-miR-4449	0.015	0.029	0.020	1.926	0.014	0.9

(d) Group C

Name of miRNA	Average		p-value (t-test)	Fold change	Difference ((Glaucoma) -(Control))	AUC
	Control	Glaucoma				
hsa-miR-1587	0.084	0.040	0.005	0.483	-0.043	0.9
hsa-miR-486-3p	0.063	0.028	0.008	0.436	-0.036	0.8
hsa-miR-3185	0.066	0.042	0.014	0.642	-0.024	0.8
hsa-miR-940	0.083	0.025	0.009	0.303	-0.058	0.8
hsa-miR-3652	0.040	0.013	0.015	0.335	-0.026	0.9
hsa-miR-3135b	0.084	0.052	0.020	0.626	-0.031	0.8
hsa-miR-5572	0.077	0.034	0.018	0.437	-0.044	0.8
hsa-miR-4640-5p	0.058	0.031	0.035	0.525	-0.028	0.7

* AUC = area under the curve.

Figure 3 | (a) Venn diagram of the miRNAs detected in all control patients and all glaucoma patients. We detected 121 miRNAs in all 20 patients (group A), 14 miRNAs in all glaucoma patients, but not in all control patients (group B), and 37 miRNAs in all control patients, but not in all glaucoma patients (group C). (b) List of 18 miRNAs with a significant difference in glaucoma and control patients, screened from group A. (c) List of 3 miRNAs up-regulated in glaucoma patients and exhibiting a significant difference in the glaucoma and control patients, screened from group B. (d) List of 8 miRNAs down-regulated in glaucoma patients and exhibiting a significant difference in glaucoma and control patients, screened from group C.

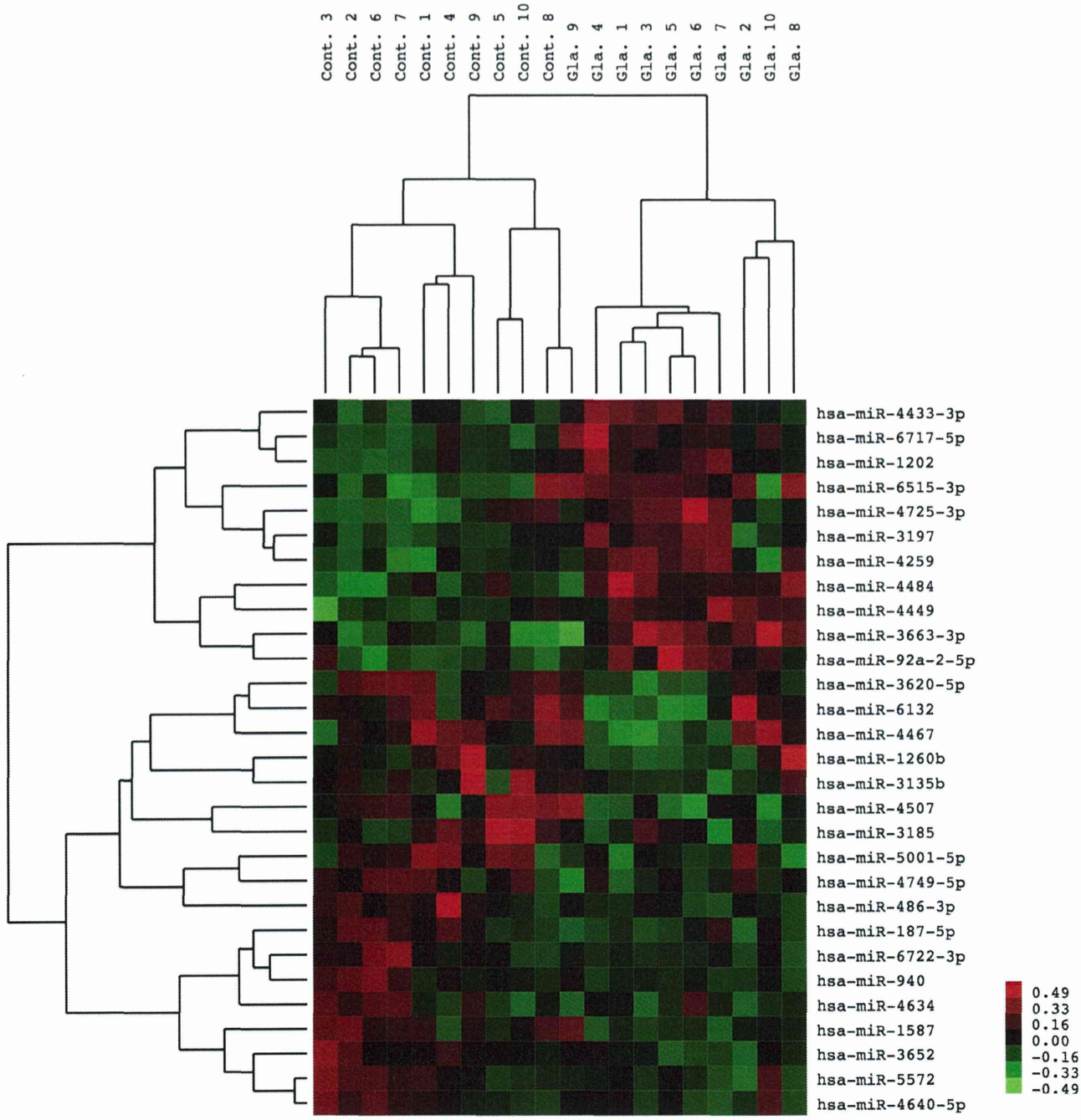


Figure 4 | Cluster analysis of selected 29 miRNA markers for glaucoma.

(2) tissue development, neurological diseases, and auditory diseases (Table 3). The top 2 predicted canonical pathways were (1) protein kinase A signaling and (2) calcium signaling (Table 4).

Discussion

The AH is an attractive source of novel miRNA biomarkers of ocular diseases, because of its accessibility, specificity, independence from other organs and novelty. Even though many researchers have reported various risk factors and biomarkers for ocular diseases epidemiologically and genetically[29–35], studies of AH RNA are rare. The primary reason is that the AH is not a cellular sample, and the amount of total RNA in the AH is so small that it is undetectable with the usual spectophotometrical procedures. Even against such a challenging background, Dunmire JJ *et al.* reported a preliminary result suggesting that miRNAs exist in the AH in 2013[14], which raised interest in further study of AH RNA profiles and their use as ocular disease biomarkers.

In the present study, we evaluated the concentration of total RNA in the AH (14–85 ng/ml) for the first time, and found that it is similar to human breast milk (9.7–228.2 ng/ml) as reported by Kosaka N. *et al.*[10], but less than in other body fluids (e.g. amniotic fluid, tears, seminal fluids, and so on) as reported by Jessica AW[11]. Additionally, the size distribution of RNA in the AH (<200 nt, Fig. 1a) is shorter than in breast milk (<300 nt). The high miRNA/small RNA purity in the AH may compensate for miRNAs detection and relevancy as a source of biomarkers.

Table 2 | Molecules predicted by DIANA-microT (v3.0) to be targeted by more than one of the miRNAs that were significantly up- or down-regulated in the glaucoma patients. AUC: Area under the curve

	Average					
Name of miRNA	Control	Glaucoma	p-value (t-test)	Fold change	Difference ((Glaucoma)-(Control))	AUC
hsa-miR-4507	0.095	0.069	0.005	0.728	−0.026	0.85
hsa-miR-3620-5p	0.211	0.131	0.006	0.622	−0.080	0.88
hsa-miR-4484	0.071	0.117	0.008	1.639	0.045	0.84
hsa-miR-5001-5p	0.230	0.166	0.011	0.718	−0.065	0.82
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hsa-miR-4467	0.972	0.768	0.013	0.790	−0.204	0.82
hsa-miR-3663-3p	0.135	0.193	0.015	1.432	0.058	0.86
hsa-miR-187-5p	0.089	0.047	0.020	0.524	−0.042	0.80
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hsa-miR-6722-3p	0.213	0.110	0.027	0.517	−0.103	0.78
hsa-miR-4725-3p	0.049	0.078	0.029	1.588	0.029	0.76
hsa-miR-1202	0.044	0.061	0.032	1.388	0.017	0.84
hsa-miR-3197	0.258	0.375	0.045	1.454	0.117	0.78
hsa-miR-4749-5p	0.159	0.126	0.046	0.794	−0.033	0.75
hsa-miR-1260b	0.683	0.450	0.046	0.659	−0.233	0.85
hsa-miR-4634	0.185	0.136	0.050	0.738	−0.048	0.75

One of the advancements of our study was the larger number of tested miRNAs than in any other study of miRNAs in other body fluids (e.g. 264 targets with a PCR array for an AH mixture from 5 cataract patients[14], 723 targets with a micro array for breast milk[10], and 706 targets with a PCR array for 12 body fluids[11]). Detected miRNAs in an individual AH sample reached five hundred on average, which was approx. 5 times more than that were detected in a mixture of 5 AH samples. Furthermore, our study is the first to reveal that individual miRNA profiles vary for every patient, and that the number of miRNA(s) detected in common was limited to 121 (26%, in average) in 20 patients.

This is the first report to identify a number of miRNAs in the AH, and compare them between glaucoma and control patients (cataract, and ERM) (Figure 3b, c, and d). We also found a higher AUC for these miRNAs in the glaucoma patients. In addition, Hsa-let-7b-3p was revealed as having the best odds ratio for glaucoma (7 glaucoma patients and 0 control patients) in this study (Supplementary Table S2 online). Furthermore, combining these markers has the potential to increase the sensitivity of glaucoma diagnosis (Figure 4).

We also found that miRNA profiles had the potential to reveal the stage of pathology, which is not possible with a genome analysis. For example, Let-7b miRNA, which had the best odds ratio for glaucoma in this study, has been reported to be an age-related marker of cataracts when expressed in the lens epithelium[36], as well as a down-regulation marker of tumorigenesis and retinoblastomas[37]. Additionally, bioinformatics predicted which molecular targets (Table 2, Supplementary Table S6 online), molecular networks (Table 3), and canonical pathways (Table 4) were related to the detected miRNA biomarker candidates. Several predicted target molecules such as ENFB3, ESRRG, and BCL2 family (Table 2, Supplementary Table S7 online) have been already reported as glaucoma-related-molecules in clinical study and/or animal model[38–40]. The predicted data obtained in this study, may lead to useful findings on the pathology of glaucoma and may reveal novel therapeutic targets.

Glaucoma can be categorized into primary open angle glaucoma (POAG), normal tension glaucoma (NTG), primary angle closure glaucoma (PACG), and other subtypes, and their pathological conditions are extremely varied. In addition, medication for patients has a potential to influence in AH miRNA profiles. In a future study, we expect to find miRNA biomarkers that can diagnose these glaucoma subtypes, pathological conditions, and pharmacological effects. We also expect that discovery of new miRNAs will be dramatically accelerated by the development of new analyzer machines, such as next-generation sequencers[28,41–43]. Exhaustive analysis of miRNAs is expected to be carried out periodically to update our knowledge of biomarkers and therapeutic targets.

In summery, we found short RNA in the AH of our patients, and profiling with a microarray system identified several candidate miRNAs related to glaucoma. Additionally, more than a hundred miRNAs were found in most individual AH miRNA profiles that had potential as biomarkers and therapeutic targets for various ocular and other diseases.

Methods

AH sampling. This research (University Hospital Medical Information Network; UMIN Study ID N.: UMIN000011121) was conducted in accordance with the Declaration of Helsinki and Tohoku University Medical School's Institutional Review Board approved the protocol (2012-1-546). Informed consent was obtained from patients undergoing glaucoma, cataract, and epiretinal membrane surgery at Tohoku University Hospital, Sendai, Miyagi, Japan. AH was collected from patients who met the inclusion criterion of having had no history of cancer, asthma, and ocular diseases other than glaucoma, cataract, and epiretinal membrane.

The enrolment and grouping criteria were listed as follows: (1) POAG was diagnosed as glaucomatous optic neuropathy on fundoscopic examination, characteristic visual field defects on the Humphrey Field Analyzer according to the Anderson-

Table 3 | Top 5 molecular networks predicted by an IPA analysis of miRNA targets

Score	Focus Molecules	Top Diseases and Functions
48	27	RNA Post-Transcriptional Modification, Developmental Disorder, Hereditary Disorder
37	22	Tissue Development, Neurological Disease, Auditory Disease
33	20	Connective Tissue Disorders, Dermatological Diseases and Conditions, Gastrointestinal Disease
32	22	Organ Morphology, Renal and Urological System Development and Function, Cellular Assembly and Organization
30	19	Cellular Assembly and Organization, DNA Replication, Recombination, and Repair, Cell Morphology