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Evaluation of circulating miRNAs in wet age-related macular degeneration

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Purpose: In the present study, we aimed to investigate the changes in plasma miRNA in patients with wet age-related macular degeneration.
Methods: The expression profiles of 384 miRNAs in plasma from 33 patients (22 male, 11 female) who were diagnosed with wet age-related macular degeneration with fundus examination, fundus fluorescein angiography, and optical coherence tomography and 31 controls (17 male, 14 female) were evaluated using high-throughput quantitative real-time PCR.
Results: Our results demonstrated that the expression level of five miRNAs (miR-17-5p, miR-20a-5p, miR-24-3p, miR-106a-5p, and miR-223-3p) was significantly upregulated in patients with age-related macular degeneration when compared to the control group ($p<0.05$). The expression level of 11 miRNAs (miR-21-5p, miR-25-3p, miR-140-3p, miR-146b-5p, miR-192-5p, miR-335-5p, miR-342-3p, miR-374a-5p, miR-410, miR-574-3p, and miR-660-5p) was significantly downregulated in patients ($p<0.05$). In addition, ten miRNAs (miR-26b-5p, miR-27b-3p, miR-29a-3p, miR-139-3p, miR-212–3p, miR-324-3p, miR-324-5p, miR-532-3p, miR-744-5p, and miR-Let-7c) were expressed only in the patient group.
Conclusions: Our results suggest that plasma miRNA levels may change in wet age-related macular degeneration. These molecules may have an important therapeutic target in patients who are unresponsive to antivascular endothelial growth factor therapy. However, further studies must be conducted for possible effects of miRNAs in vascular disorders of eye such as age-related macular degeneration.

Age-related macular degeneration (AMD) is the leading cause of irreversible vision loss in people over the age of 65 in the developed world [1]. In addition, AMD has become an important public health problem. AMD is divided into two clinical forms by international consensus: the dry type and the wet type [2]. “Dry” type is characterized by slow and progressive degeneration of the RPE and Bruch’s membrane. However, the development of choroidal neovascular membrane (CNVM) is characteristic of the “wet” type and causes fluid leakage, RPE detachment, hemorrhage, exudation, and scarring [2].

Many studies are being conducted to understand the molecular mechanisms related to wet AMD and the role of genetics and environmental risk factors. Although the molecular mechanisms of wet AMD remain unclear, it is postulated that oxidative stress, inflammation, and angiogenesis are important factors in pathogenesis [2,3]. Vascular endothelial growth factor (VEGF) has been shown to play a causal role in the development of CNVM [4]. Studies have demonstrated that anti-VEGF agents (bevacizumab, ranibizumab) are efficacious in treating CNVM in wet AMD, but they have limited efficacy [5]. Krebs et al. [6] showed that about 15% of patients with AMD did not respond sufficiently to anti-VEGF treatment. The molecular mechanisms are not known, but new molecules such as miRNAs may play a role in the pathophysiology of wet AMD.

MiRNAs are small-noncoding molecules that have a critical function in gene expression [7]. It has been predicted that 30% of human genes may be regulated by miRNAs [8]. Studies have also shown that miRNAs play an important role in the development of the eye, ocular homeostasis, and ocular diseases [9]. A group of miRNAs has also been shown to play critical roles in different pathways in a laser-induced CNVM mouse model [10]. miR-31, miR-150, and miR-184 are significantly decreased in an ischemia-induced mouse model of retinal neovascularization and in a laser-induced mouse model of choroidal neovascularization in the absence of ischemia [11]. Intraocular injection with pre-miR-31 or pre-miR-150 significantly reduced the size of the choroidal neovascular lesions. A recent study also demonstrated that knockdown of miR-23 and miR-27, which downregulate the antiangiogenic factors Sprouty2 and semaphorin 6A (Sema6A), is protective against laser-induced choroidal neovascularization [10]. miR-23a has also been found to be decreased in RPE cells from AMD donor eyes. In ARPE-19 cells, antisense-mediated

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inhibition of miR-23a reduced cell growth, and adding a miR-23a mimic reduced hydrogen peroxide–induced oxidative damage and Fas-mediated apoptosis [12].

miRNAs may have important therapeutic implications in wet AMD, but further studies must be conducted for possible effects of miRNAs in vascular disorders of the eye such as age-related macular degeneration. We aim to investigate the differential expression of miRNAs between patients with wet AMD and healthy controls and to assess the diagnostic potential of plasma miRNAs as biomarkers for early detection of AMD. Our study is the first clinical study in the literature regarding this aspect.

METHODS

Thirty-three patients (22 men, 11 women) who were newly diagnosed wet AMD with fundus examination, fundus fluorescein angiography (FFA), and optical coherence tomography (OCT) referred to Mersin University, Department of Ophthalmology during September 2012–April 2013 were included in the study. The control group consisted of 31 people (17 men, 14 women). Controls were selected among healthy people with no history of cancer, chronic degenerative neurologic disease, diabetes, atopy, or autoimmune diseases and without ocular, systemic pathology, or allergies in general. All subjects were systematically interviewed about their current and lifetime smoking status and occupational risk factors for AMD. Peripheral blood samples of the patient and control groups were obtained and analyzed for expression of 384 miRNAs in plasma. The investigations were approved by the Medical Ethical Review Committee of the Academic Hospital of Mersin University (2012/255), and informed consent was obtained from all patients and controls according to the Declaration of Helsinki II (1975, revised 1983), and the study adhered to the ARVO statement on human subjects.

Plasma preparation and RNA isolation: With venipuncture, 3 ml venous blood was collected in sterile siliconized 7.5% EDTA-containing tubes. Plasma samples were collected via centrifuging the EDTA blood samples at 2280 ×g for 15 min within 2 h of collection. Plasma samples were transferred to a clean microcentrifuge tube followed by a second high-speed centrifugation step at 15,000 ×g for 5 min at 10 °C to remove cell debris and fragments. The plasma samples were aliquoted and stored at −80 °C until RNA extraction. Total RNA (including miRNAs) was extracted from plasma by using the High Pure miRNA Isolation Kit (Roche diagnostic GmbH, Mannhein, Germany) according to the manufacturer's instructions and then stored at −80 °C for further processing.

Reverse transcription reaction: Total RNA samples (2 µl) were reverse transcribed using the TaqMan MicroRNA Reverse Transcription Kit in combination with the Megaplex RT Primer Human Pool Set A (Applied Biosystems, Foster City, CA), allowing simultaneous reverse transcription of 384 miRNAs. Briefly, 2 µl of total RNA was supplemented with Megaplex RT primer mix (10X), RT buffer (10X), MultiScribe Reverse Transcriptase (50 U/µl), dNTPs with dTTP (100 mM), $MgCl_2$ (25 mM), and RNase inhibitor (20 U/µl) in a total reaction volume of 5 µl. The reverse transcription (RT) reaction was used (40 cycles of 16 °C for 2 min, 42 °C for 1 min and 50 °C for 1 s, followed by a final reverse transcriptase inactivation at 85 °C for 5 min). cDNA samples were kept at −80 °C until PCR analysis.

Preamplification of cDNA: For preamplification, 2 µl of cDNA samples were transferred into a clean 96-well plate, and 8 µl of DNA suspension buffer was added on the top of the cDNAs and mixed by pipetting up and down five to six times. One fifth of the diluted RT product (2 µl) was preamplified using the Applied Biosystems TaqMan PreAmp Master Mix (2X) and Megaplex PreAmp Primers (5X) in a 5 µl PCR reaction. The primer pool consisted of forward primers specific for each of the 384 miRNAs and a universal reverse primer (Applied Biosystems). The preamplification cycling conditions were as follows: 95 °C for 10 min, 55 °C for 2 min and 75 °C for 2 min followed by 14 cycles of 95 °C for 15 s, and 60 °C for 4 min.

miRNA expression determination using microfluidic high-throughput qRT-PCR: Each chip profiled 64 samples for the expression level of 96 miRNAs. Preamplified cDNA samples were diluted with low-EDTA (0.1 mM) TE buffer (1:5). About 490 µl TaqMan Universal PCR Master Mix (no AmpErase UNG; Applied Biosystems) and 49 µl (20X) GE sample loading reagent (Fluidigm, San Francisco, CA, PN 85,000,746) were mixed, and 3.85 µl was pipetted into a 96-well plate; then 3.15 µl of diluted preamplified cDNA was added into each well and mixed. Then 5 µl of this mixture was pipetted into the sample inlets of a 96.96 dynamic array (Fluidigm), and 4.5 µl of miRNA TaqMan probe and primers (Applied Biosystems) were pipetted into assay inlets of a 96.96 dynamic array. The Biomark IFC controller HX (Fluidigm) was used to distribute the assay mix and sample mix from the loading inlets into the 96.96 dynamic array reaction chambers for quantitative real-time PCR (qRT-PCR) with the Fluidigm integrated microfluidic circuit technology. qRT-PCR was performed using the high-throughput Biomark real-time PCR system (Fluidigm). The cycling conditions were as follows: The thermal mix protocol was followed by 50 °C for 2 min, 70 °C for 30 min and 25 °C for 5 min. Then the UNG and Hotstart protocol was followed by 50 °C for 2 min and 95 °C for 5 min. Finally, the PCR cycle was followed by 40 cycles

of 95 °C for 15 s (denaturation) and 60 °C for 60 s (annealing). The real-time qPCR analysis software provided amplification curves, color-coded heat maps, and the cycle threshold (Ct). Threshold and linear baseline correction were automatically calculated for the entire chip.

Normalization and relative quantification of plasma miRNA expression: To eliminate the normalization problem for miRNA expression in plasma, depending on the absence of stable RNA, we used the global mean normalization method for normalizing plasma miRNA expression. The miRNA expression data were normalized according to the global mean normalization strategy [13]. The global mean normalization of the miRNA qRT-PCR data was performed with GenEx Professional 5 software (MultiD Analyses AB, Goteborg, Sweden). The relative expression of miRNAs was calculated with the comparative ΔCT (ΔΔCT) method. Fold change (FC) was calculated with equation $2^{-\Delta\Delta Ct}$ [14].

RESULTS

A total of 64 individuals were enrolled in the present study, including 33 patients with AMD and 31 controls. Table 1 presents the characteristics of the subjects. The mean age of the study group was 72.12±8.75 years and the control group 62.68±7.13 years. The mean age was significantly different between the groups ($p<0.05$), but the gender distribution and the hypertension profile were similar in the groups ($p>0.05$; Table 1).

Plasma samples from 33 patients with AMD and 31 control subjects were examined for the expression of the 384 miRNAs using high-throughput qRT-PCR. Forty-nine miRNAs were expressed in both groups (Table 2). Among these miRNAs, 16 miRNAs were aberrantly expressed, of which 11 miRNA were significantly downregulated and five upregulated in the patient group comparing with the control group (Figure 1, Table 3). In addition, we determined that ten miRNAs (miR-26b-5p, miR-27b-3p, miR-29a-3p, miR-139-3p, miR-212-3p, miR-324-3p, miR-324–5p, miR-532-3p, miR-744-5p, and miR-Let-7c, respectively) were expressed only in the patient group.

Statistical analysis: Statistical analysis was performed with the SPSS software package, version 17.0 for Windows (SPSS, Chicago, IL). Power calculations for testing the sample size were performed using the PASS software package program (NCSS, LLC, UT, version 11.0 for Windows; desired study power, 80%; α error=0.05, two-tailed). Chi-square ($\chi 2$) was used to evaluate the distribution of hypertension and gender among the patients and the control subjects. The age of both groups was compared with an independent sample *t* test. Expression data were controlled for normal distribution with the Shapiro-Wilk test. According to the test results, all data were not normally distributed. The Mann–Whitney U-test was used to detect differences in the expression of plasma miRNAs between the patients and healthy subjects. Results are expressed as mean ± standard deviation (SD). A p value of <0.05 was considered significant.

DISCUSSION

Although various risk factors have been reported, the pathophysiology of wet AMD remains unclear. In recent studies, miRNAs have been found to play a critical role in pathological angiogenesis, oxidative stress, and inflammation, which have important roles in this disease. However, studies investigating

Table 1. Characteristic of control and patients with AMD.

Characteristic	Control (n=31)	AMD (n=33)	P value
Sex			
Male	17	22	0,443
Female	14	11	
Age (years)	62,68±7,13	72,12±8,75	0,001*
Hypertension			
Yes	16	17	0,994
No	15	16	
Involvement			
Bilateral	—	5	
Unilateral	—	28	
Cataract operation			
Phakic	—	24	
Pseudophakic	—	9	

Table 2. Expression of plasma miRNAs in patients with AMD as compared to control group.

miRNA	Control	AMD	P value	FC
	ΔCt (Mean ±SD)	ΔCt (Mean ±SD)		
miR-19a-3p	5,76±1,16	6,2±0,93	0,252	−1,35
miR-140–3p	5,73±2,11	7,22±0,86	0,029*	−2,81
miR-19b-3p	−0,26±1,11	−0,23±1,31	0,800	−1,02
miR-20a-5p	1,07±1,5	0,06±0,94	0,035*	2,02
miR-20b-5p	6,24±1,29	5,98±1,27	0,487	1,20
miR-103a-3p	6,74±2,11	6,05±2,18	0,770	1,62
miR-130a-3p	3,17±1,16	4,53±1,09	0,125	−2,57
miR-21–5p	6,66±1,05	8,29±1,42	0,045*	−3,09
miR-30c-5p	0,87±0,96	1,46±1,17	0,114	−1,50
miR-106a-5p	3,23±1,38	1,44±1,27	0,001*	3,45
miR-106b-5p	2,12±1,04	1,43±1,45	0,141	1,62
miR-133a	4,93±0,96	6,68±2,07	0,182	−3,34
miR-146a-5p	2,82±1,71	3,42±1,37	0,201	−1,52
miR-24–3p	−0,66±0,90	0,00±1,06	0.045*	1,58
miR-122–5p	4,6±2,43	6,18±2,43	0,240	−2,98
miR-25–3p	−0,68±0,9	0,2±1,28	0,021*	−1,84
miR-146b-5p	4,67±1,11	7,79±1,44	0,006*	−8,68
miR-26a-5p	6,1±1,1	6,82±1,56	0,409	−1,65
miR-92a-3p	−2,33±1,62	−2,57±0,98	0,924	1,18
miR-93–5p	4,58±1,05	4,35±1,09	0,369	1,17
miR-148a-3p	8,49±1,34	6,89±1,47	0,245	3,02
miR-27a-3p	4,9±1,41	5,37±2,09	0,610	−1,38
miR-125b-5p	2,91±1,21	4,75±1,99	0,527	−3,59
miR-16–5p	1,23±1,1	1,61±0,92	0,155	−1,30
miR-126–3p	1,09±1,39	1,23±0,96	0,290	−1,10
miR-17–5p	1,96±0,95	0,49±1,23	0,001*	2,77
miR-139–5p	3,19±1,8	4,62±1,43	0,092	−2,71
miR-192–5p	6,11±2,22	7,93±1,01	0,007*	−3,54
miR-203	3,49±0,96	4,93±2,92	0,465	−2,72
miR-296–5p	5,78±1,44	7,13±1,29	0,111	−2,55
miR-150–5p	−1,03±1,49	−0,94±1,4	0,661	−1,06
miR-152	4,56±1,22	3,71±1,33	0,380	1,81

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miRNA	Control	AMD	P value	FC
	ΔCt (Mean ±SD)	ΔCt (Mean ±SD)		
miR-193b-3p	3,4±1,98	5,65±1,81	0,063	−2,76
miR-331–3p	5,65±1,79	5,57±1,17	0,655	1,05
miR-335–5p	4,78±0,8	7,59±1,63	0,019*	−7,00
miR-197–3p	5,97±1,14	7,28±1,58	0,089	−2,47
miR-221–3p	4,73±1,71	3,45±2,96	0,309	2,43
miR-342–3p	4,01±1,49	5,42±1,06	0,001*	−2,65
miR-190a	2,68±1,75	2,33±1,09	0,427	1,28
miR-223–3p	−1,89±1,04	−2,61±2,24	0,013*	1,65
miR-191–5p	3,02±1,83	2,89±1,08	0,797	1,10
miR-374a-5p	−1,89±2,6	1,81±6,41	0,026*	−12,92
miR-484	−0,85±1,43	−1,01±1,71	0,569	1,12
miR-375	2,64±1,89	5,37±2	0,134	−2,66
miR-520d-5p	4,11±2,08	5,6±1,47	0,193	−2,81
miR-451a	−2,91±1,19	−3,31±1,04	0,104	1,32
miR-410	−2,67±2,54	0,63±2,97	0,001*	−9,84
miR-660–5p	4,3±1,13	6,06±1,39	0,002*	−3,39
miR-574–3p	3,13±1,54	4,42±0,97	0,007*	−2,44

SD; standard deviation. FC; fold change. ΔCt; delta threshold cycle.

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Table 3. Upregulated and downregulated miRNAs, which target the VEGF-A gene.

miRNAs	miRNA	Target gene
Up Regulated miRNAs	miR-20b-5p	VEGF-A
	miR-24–3p	VEGF-A
	miR-106a-5p	VEGF-A
	miR-17–5p	VEGF-A
Down Regulated miRNAs	miR-335–5p	VEGF-A

In this study, upregulated and downregulated miRNAs which target the VEGF-A gene was determined by Mirwalk and Mirtarbase.

the relationship between disease and miRNAs have remained at the cellular or animal models [15,16]. To the best of our knowledge, a clinical study has not been conducted. In our clinical study, we aimed to analyze the relationship between wet AMD and miRNA. This aspect of the study has the distinction of being the first clinical study in the literature. If this relationship can be clearly demonstrated, early diagnosis and screening tests can be developed, and new treatment alternatives can be demonstrated in individuals genetically predisposed. We have included updated names for miRNAs in parentheses according to the miRBase database.

Xu et al. [17] found that miR-140 (new name: miR-140-3p) is highly expressed in the adult retina but is not detectable in RNA from the brain or other tissues. In our study, we found that miR-140-3p expression decreased significantly in the patient plasma (FC: 2.81, p=0,029). Based on this study, we believe miR-140-3p plays a role in normal functioning of the retina, and this may cause degradation in normal functioning of the retina.

In vitreous humor from patients with ocular diseases (retinal detachment, macular hole, and uveal melanoma), miR-374a (new name: miR-374a-5p) was low expressed compared to the serum [18]. In our study, miR-374a-5p was significantly downregulated in the plasma from patients with AMD (FC: 12.92, p=0.026). Decreased expression of miR-374-5p may play an active role in the development of wet AMD like the other listed ocular diseases.

Studies have shown that although some miRNAs are angiogenic, others are antiangiogenic. Shi et al. [19] determined that decreased miR-223 (new name: miR-223-3p) levels in vivo were also associated with a marked increase in angiogenesis in the murine retina and hind limb. In addition, Shen et al. [11] determined that miR-106a (new name: miR-106a-5p) was substantially increased in the mice model of ischemia-induced retinal neovascularization. Suarez et al. [20] showed that miR-17–5p had proangiogenic activity. In our study, miR-223-2p, miR-106a-5p, and miR-17–5p significantly increased in the plasma of patients with AMD compared to the control group (FC: 1.65, p=0.013; FC: 3.45, p=0.001; FC: 2.77, p=0.0001, respectively). An increase in these miRNAs

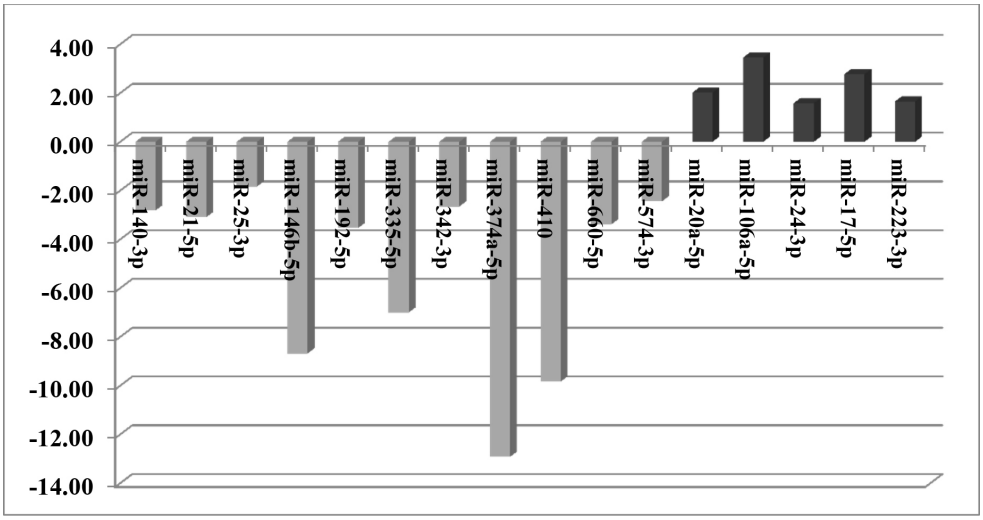

Figure 1. Fold change of miRNAs is significantly downregulated and upregulated in patients with age-related macular degeneration compared to the control group.

can cause wet AMD by triggering angiogenesis. In the future, if the relationship between them is understood more clearly, molecules that block these miRNAs can be developed.

Sabatel et al. [21] demonstrated that overexpression of miR-21 (new name: miR-21-5p) represses laser-induced CNV in mice. They determined that miR-21 exhibits antiangiogenic function by targeting RhoB expression in endothelial cells. Zhou et al. [22] also determined that subretinal delivery of miR-24 (new name: miR-24-3p) mimics represses laser-induced CNV in vivo. We determined that expression of miR-21-5p downregulated whereas miR-24-3p expression upregulated significantly in patient plasma (FC: 3.19, p=0.045; FC: 1.58, p=0.045, respectively). A decrease in miR-21-5p expression can cause CNVM, and an increase in miR-24-3p expression could be a compensation mechanism. Nevertheless, more studies must be conducted to investigate the relationship between them. These studies could suggest that the balance between angiogenic and antiangiogenic miRNAs favor angiogenic miRNAs.

Some studies investigated the relationship between miRNAs and VEGF. Studies indicate that miR-192 (new name: miR-192-5p) downregulated the expression of Bcl-2, Zeb2, and VEGF-A in vitro and in vivo [23]. In addition, He et al. [24] found that miR-342 (new name: miR-342-3p) expression was upregulated in VEGF-negative tumors compared with VEGF-positive tumors in breast cancer tissues. These studies showed that VEGF expression was decreased while miR-192-5p and miR-342-3p expression was increased. In our study, we determined that the expression of miR-192–5p and miR-342-3p was downregulated significantly in patient plasma (FC: 3.54, p=0.007; FC: 2.65, p=0.001, respectively). Due to the decrease in these miRNAs, VEGF expression may be increased, and we believe that pathological angiogenesis occurred because of decreased VEGF expression. Mimics of these miRNAs may be developed in the future and may be used in treatment. Hua et al. [25] investigated the relationship between miRNAs and VEGF. The researchers found that miR-20a (new name: miR-20a-5p) was downregulated in hypoxia-induced CNE cells. The researchers also observed that miR-20a and miR-92a act have antiangiogenesis activity, targeting the VEGF-A transcript. We found plasma miR-20a-5p was significantly upregulated in patients with AMD (FC: 2.02, p=0.035). Increased miR-20a-5p may compensate for the increase in miR-24-3p. Studies should be conducted to evaluate the relationship between miRNAs and VEGF.

miRNAs are known to play a role in the inflammation process, which is also believed to be an element in the development of wet AMD. Kutty et al. [26] clearly showed that miR-146b-5p was expressed in human RPE cells in culture and the expression was highly induced by proinflammatory cytokines. These results showed for the first time that miR-146b-5p expression was regulated by interferon (IFN)-γ, potentially via the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway. In the present study, miR-146b-5p was significantly decreased in the plasma of patients with AMD compared to the control subjects (FC: 8.68, p=0.006). We suggest that miR-146b-5p could play a role in inflammatory processes underlying AMD or other retinal degenerative diseases through their ability to negatively regulate the nuclear factor-κB pathway by targeting the expression of interleukin-1 receptor-associated kinase 1 (IRAK1).

Bai et al. [27] showed that in aging mesangial cells, the expression level of miR-335 (new name: miR-335-5p) and miR-34a was significantly upregulated, whereas the predicted target genes (superoxide dismutase-2 [SOD2, OMIM 147460] and thioredoxin reductase-2 [TXNRD2, OMIM 606448]) of miR-335 and miR-34a were markedly downregulated. miR-335-5p and miR-34a could inhibit superoxide dismutase-2 and thioredoxin reductase-2 expression through binding to the corresponding binding sites in the 3′- untranslated regions (UTRs) of the superoxide dismutase-2 and thioredoxin reductase-2 genes. In this study, we detected down expression of miR-335–5p in AMD (FC: 7.0, p=0.019). This decreased expression might have developed to control oxidative stress in a more comfortable way.

Previous studies on the search for plasma/serum miRNA-based disease biomarkers generally focused on individual disease-specific miRNAs. Therefore, although a specific miRNA plasma/serum alone may help to distinguish between patients and healthy controls, a panel of miRNAs has greater potential to offer a more specific diagnosis. In the validation phase of the present study, we determined ten miRNAs that showed AMD-specific expression (miR-26b-5p, miR-27b-3p, miR-29a-3p, miR-139-3p, miR-212-3p, miR-324-3p, miR-324-5p, miR-532-3p, miR-744-5p, and miR-let-7c).

N-(4-hydroxyphenyl)-retinamide (4-HPR), a retinoic acid derivative, induces reactive oxygen species generation and apoptosis in cultured human RPE cells treatment increased the expression of miR-26b (new name: miR-26b-5p) in ARPE-19 cells [28]. In our study, we found that miR-26b-5p was expressed only in the patient group. Oxidative stress in RPE cells has an important role in the development of wet AMD, and expression of this miRNA might be a response to oxidative stress in patients. Early detection or screening tests can be developed if the relationships between them are defined clearly.

Kuehbacher et al. [29] showed that inhibition of miR-27b significantly reduces endothelial cell sprouting in an in vitro setting. Urbich et al. [30] showed that miR-27a/b promotes angiogenesis by targeting endogenous angiogenesis inhibitor semaphorin 6A (SEMA6A) and controlling endothelial sprouting. Biyashev et al. [31] demonstrated that delta like ligand 4 (Dll4) and sprouty homolog 2 (Spry2) were targets of miR-27b and therefore the effectors of miR-27b action on the angiogenic switch. We also determined expression of this miRNA only in the patient group. These studies showed that miR-27-3p and angiogenesis were closely associated. Another study showed that miRNA-29a (new name: miR-29a-3p) targeted VEGF-A and inhibited tumor growth [32]. In our study, we found that miR-29a-3p was expressed only in the patient group. This expression might have occurred to inhibit VEGF-A, which is known to have increased expression in wet AMD.

In our study, we determined that miR-25-3p, miR-410, miR-574-3p, and miR-660-5p expression was significantly decreased in the patients' plasma while miR-139-3p, miR-212-3p, miR-324-3p, miR-324-5p, miR-532-3p, miR-744-5p, and miR-let-7c were expressed only in the patient group. To date, many studies with these miRNAs are limited to cancer patients [33-38]. The relationship between these miRNAs and other disease has not been clearly revealed. These miRNAs might have relationships with angiogenesis, inflammation, and oxidative stress, which are responsible for the development of cancer. Therefore, we believe that these miRNAs might also be responsible for the development of wet AMD.

In our study, the mean age of the patient group was significantly higher than that of the control group. This might be a disadvantage for our study since miRNA expression levels can change with advancing age. At the same time, incidence of AMD and systemic diseases increases with advancing age. Thus, finding healthy older individuals without ocular and systemic diseases can be difficult.

In conclusion, these differentially expressed miRNAs might be novel targets for further investigation of the molecular pathogenesis and management of AMD. We suggest that these miRNAs may be potential candidates for novel biomarkers in wet AMD. Our results should be confirmed with larger studies, and further studies for investigating the biologic function and origin of circulating plasma miRNAs should be conducted in the future.

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