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Identification of Novel Serum MicroRNAs in Age-Related Macular Degeneration

Hanan ElShelmani[1], Michael A. Wride[1], Tahira Saad[2], Sweta Rani[3], David J. Kelly[4], and David Keegan[2]

[1] Ocular Development and Neurobiology Research Group, Zoology Department, School of Natural Sciences, University of Dublin, Trinity College Dublin, Dublin, Ireland
[2] Mater Misericordiae University Hospital, Dublin, Ireland
[3] Department of Science, Waterford Institute of Technology, Waterford, Ireland
[4] Zoology Department, School of Natural Sciences, University of Dublin, Trinity College Dublin, Dublin, Ireland

Correspondence: David Keegan, Mater Misericordiae University Hospital, Eccles St., Dublin 7, Ireland. e-mail: dkeegan@mater.ie

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Purpose: To identify circulating microRNAs (miRNA) associated with age-related macular degeneration (AMD). Thus differentially expressed serum miRNA could be used as AMD biomarkers.

Methods: This study involved total RNA isolation from sera from patients with atrophic AMD (n = 10), neovascular AMD (n = 10), and age- and sex-matched controls (n = 10). A total of 377 miRNAs were coanalyzed using array technologies, and differentially regulated miRNAs were determined. Extensive validation studies (n = 90) of serum from AMD patients and controls confirmed initial results. Total RNA isolation was carried out from sera from patients with atrophic AMD (n = 30), neovascular AMD (n = 30), and controls (n = 30). Fourteen miRNAs from the discovery dataset were coanalyzed using quantitative real-time polymerase chain reaction (qRT-PCR) to validate their presence.

Results: Unsupervised hierarchical clustering indicated that AMD serum specimens have a different miRNA profile to healthy controls. We successfully identified and validated the differentially regulated miRNAs in serum from AMD patients versus controls. The biomarker potential of three miRNAs (miR-126, miR-19a, and miR-410) was confirmed by qRT-PCR, with significantly increased quantities in serum of AMD patients compared with healthy controls.

Conclusions: Increased quantities of miR-126, miR-410, and miR-19a in serum from AMD patients indicate that these miRNAs could potentially serve as diagnostic AMD biomarkers. All three miRNAs significantly correlated with AMD pathogenesis.

Translational Relevance: The discovery of new AMD miRNA may act as biomarkers in evaluating AMD diagnosis and prognosis.

Introduction

Age-related macular degeneration (AMD) is a multifactorial disease, associated with a complex interaction of genetic and environmental factors.[1] The early stage of AMD is characterized by dysfunction of retinal pigment epithelium (RPE) cells, with pigment changes and drusen deposition, which are essential for the function of the photoreceptors, whereas the late stage disease develops neovascularization and/or geographic atrophy with a significant loss of vision.[2,3]

MicroRNAs (miRNAs) are small noncoding RNA molecules that have shown a controlling effect on the expression of several genes.[4] Therefore it is suggested that miRNAs control a variety of normal physiological and pathological processes.[1,5] Some in vivo and in vitro studies suggested that numerous miRNAs are associated with the pathological process involved in AMD and diabetic retinopathy, such as pathological angiogenesis, oxidative stress, and inflammation, which suggests that these miRNAs might be potential therapeutic targets.[1,6,7] miRNAs provide promising biomarkers because of their significant stability

Table 1. miRNAs Biomarkers in AMD

Study	Tissue	AMD Phenotype	Predominant miRNA Expression Dry/Wet AMD
Romano et al.[13]	Serum	AMD	miR-9, miR-23a, miR-27a, miR-34a, miR-126, and miR-146a
Szemraj et al.[4]	Serum	Dry AMD, wet AMD	Dry AMD: miR-661, miR-3121; Wet AMD: miR-4258, miR-889, let-7
Ménard et al.[14]	Plasma and vitreous humor	Wet AMD	Wet AMD: miR-146a, miR-106b, miR-152
Ertekin et al.[15]	Plasma	Wet AMD	miR-17-5p, miR-20a-5p, miR-24-3p, miR-106a-5p, and miR-223-3p
Grassmann et al.[16]	Serum	Dry AMD, wet AMD	Dry AMD: mir-424-5p;Wet AMD: mir-424-5p, mir-301-3p, mir-361-5p
Ren et al.[17]	Whole blood	Dry AMD, wet AMD	miR-27a-3p, miR-29b-3p, miR-195-5p;Wet AMD: miR-27a
Elbay et al.[18]	Exosomes	AMD	miR-486-5p, miR-626

in blood and their distinctive expression in different diseases.[8,9] Using specimens that are easily obtained, such as serum, to detect AMD biomarkers by the least invasive methods could have a large impact on clinical practice, research, and trials[10–12] Table 1.

miRNAs have been investigated as promising therapeutic targets and biomarkers for different eye disorders, various miRNAs have been identified to demonstrate significant effects in conditions such as AMD, retinoblastoma, myopia, and cataract. miR-9, miR-125b, miR-146a, and miR-155 are shown to be involved in dysregulation of the complement system, which has a potential role in the pathogenesis of AMD.[19] A previous clinical study investigated the differential expression of miRNAs in patients with neovascular AMD and healthy controls. Eleven miRNAs were significantly downregulated in the patient group (miR-21, miR-25-3p, miR-140, miR-146b-5p, miR-192, miR-335, miR-342, miR-374a, miR-410, miR-574-3p, and miR-660-5p) and five miRNAs were found to be significantly upregulated (miR-17-5p, miR-20a, miR-24, miR-106a, and miR-223) compared with the control group.[15]

The objective of this study was to investigate whether we can detect novel serum miRNAs in identification of AMD.

Methods

Case-Controlled Study Design

Clinically documented AMD and control blood donors were recruited at the Mater Misericordiae University Hospital (MMUH), Dublin. Ethics approval for the study was obtained from MMUH according to the tenets of the Declaration of Helsinki. All study participants were white people from Ireland. All patients were over age 55 years, had AMD, and received a comprehensive eye examination by a clinician (DK, TS) in the MMUH Eye Clinic and provided written informed consent. AMD patients from MMUH have been defined and graded using Age-Related Eye Disease Study Age-related Macular Degeneration classification (AREDS classification system). Patients and controls with coexisting ocular pathology and/or underlying systemic diseases, such as diabetic retinopathy, high blood pressure, and other inflammatory conditions, were excluded from the study. Blood specimens were collected after clinical examination and diagnosis. Patient identifiers were removed, and the specimens were encoded to protect donor confidentiality.

The discovery study was designed to compare miRNA profiles in a number (n = 30) of control and atrophic/neovascular-AMD serum specimens from age- and sex-matched control donors. AMD disease status was categorically (atrophic and neovascular AMD) based on fundus examination. Study population characteristics are summarized in Supplementary Table S1.

For the validation study, 90 clinically documented AMD and control (i.e., no signs or symptoms of AMD) blood donors (atrophic AMD, n = 30; neovascular AMD, n = 30; and controls, n = 30) were recruited from the Mater Private Hospital, MMUH and Waterford Institute of Technology. Some of the AMD patients were a part of the CARMA study

(Carotenoids and Co-Antioxidants in Age-Related Maculopathy).[20] CARMA study serum specimens were graded with the CARMA grading system of AMD (see Supplementary Table S2 for the CARMA grading values).

The study design was to compare a number of control, atrophic, and neovascular-AMD serum specimens. AMD disease state was categorized based on fundus examination recruited at the MMUH via AREDS classification system and Waterford Institute of Technology via the CARMA grading system of AMD.

Human Serum Preparation

Nonfasting blood specimens were collected with consent from each patient and control (time between 10 AM to 4 PM). From the collected blood samples, the red blood cells were allowed to clot naturally. These specimens were processed within 3 to 4 hours of blood draw. The tubes were centrifuged at 400 g for 15 minutes. After centrifugation, 1 to 1.5 mL of the serum was carefully removed, aliquoted, and stored immediately at −80°C.

RNA Isolation

Total RNA was isolated from 0.25 mL serum specimens using TriReagent (Sigma Aldrich, Ireland Ltd). TriReagent (0.75 mL) was added to the 0.25 mL serum and incubated at room temperature for 10 minutes. Chloroform (0.2 mL) was added to each sample and this was shaken vigorously for 15 seconds, followed by incubation at room temperature for 15 minutes. This was then centrifuged at 12,000 rpm for 15 minutes at 4°C, and the aqueous phase containing RNA (upper layer) was removed and transferred into a fresh RNAse-free 1.5 mL Eppendorf tube (Sigma Aldrich, Ireland Ltd).

Isopropanol (0.5 mL) and glycogen (final concentration 120 µg/mL) were added, and the tubes were incubated at room temperature for 5 to 10 minutes before being stored at –20°C overnight, to ensure maximum RNA precipitation. Eppendorf tubes were then centrifuged at 12,000 rpm for 30 minutes at 4°C to pellet the precipitated RNA. Taking care not to disturb the RNA pellet, the supernatant was removed, and the pellet was subsequently washed by the addition of 750 µL of 75% ethanol and vortexed. Following centrifugation at 7,500 rpm for 5 minutes at 4°C, supernatant was removed, and this wash step was repeated. The RNA pellet was then allowed to air-dry for 5 to 10 minutes, and subsequently was resuspended in 10 µL of DEPC-treated H_20 (Ambion, Inc., Austin, TX). The RNA was stored at –80°C until further analysis.

Quantity and Quality of Extracted RNA

RNA concentration and quality was measured using the Nanodrop (Thermo Fisher Scientific, Waltham, MA). Furthermore, the TaqMan MicroRNA Assays are designed to detect and accurately quantify mature miRNAs using Applied Biosystems (Life Technologies, Europe, Irish Branch, Foster City, CA) real-time PCR instruments.

MicroRNA Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

For the discovery study (n = 30), TaqMan MicroRNA Low Density Arrays (TLDA, Applied Biosystems) was selected as the platform for microRNA profiling. RNA reverse transcription was performed according to the Applied Biosystems microRNA TLDA Reverse Transcription Reaction protocol. cDNA is reverse transcribed from total RNA specimens using the Megaplex Primers and the TaqMan MicroRNA Reverse Transcription Kit. The resulting cDNA was preamplified using the TaqMan PreAmp Master Mix and Megaplex PreAmp primers. A TLDA TaqMan Real-Time Assay was set up for each specimen as follows: 450 µL of TaqMan Universal PCR Master Mix-No AmpErase UNG (Applied Biosystems, Life Technologies, Europe) were added to 9 µL of diluted PreAmp product in a 1.5 mL microcentrifuge tube containing 441 µL of nuclease-free water. A total of 100 µL of the PCR reaction mix were loaded into each port of the TLDA array. All PCR reactions for each sample were in triplicate. TLDAs were run in a 7900 HT Sequence Detection system (Applied Biosystems, Life Technologies, Europe). ABI TaqMan SDS v2.3 software was used to obtain raw C_T values. To review results, raw C_T data (SDS file format) were exported.

Identification of Consistent Normalizer (Endogenous Control) for Normalization

Normalization was performed based on the average of the assays detected in all specimens, as this is proposed to be the best normalization for qRT-PCR studies involving several assays.[21]

From analysis of 384 miRNAs in the discovery 30 sera specimen cohort, using both geNorm (available at https://genorm.cmgg.be/) and Normfinder (available at http://moma.dk/normfinder-software)

programs available through GenEx software (GenEx Software-bioMCC, Freising-Weihenstephan Munich, Germany), we chose two endogenous controls based on their relative consistent abundance in all specimens (Supplementary Fig. S1). Specifically, we choose miR-323-3p and miR-324-3p to normalize the data, as their levels were similar across all specimens (Supplementary Fig. S2).

Supporting this choice, miR-323-3p and miR-324-3p were recommended by Applied Biosystems (TLDA manufacturer) and used in previous serum/blood studies as endogenous references.[22–25]

Selection Criteria of miRNA Targets

miRNA targets selected for further validation were based on the following criteria: the target miRNA should be present in patients' sera, absent in control, relative quantification (RQ) $\geq$1.5 for upregulated miRNAs, common among >70% of the AMD patients, and previously published in relation to pathways or pathological processes involved in AMD pathology.

Data Analyses

Raw C_T data (SDS file format) were exported from the Plate Centric View, Applied Biosystems, Life Technologies, Europe, Irish Branch and fold change values were calculated according to the following formula using Excel (Microsoft Corp., Redmond, WA):

$$\Delta CT = CT - CT_{(endogenous\ control)}$$

$$\Delta\Delta CT = \Delta CT - \Delta CT_{control}$$

$$Fold\ change\ (RQ\ value) = 2^{-\Delta\Delta CT}$$

Data are given as mean ± standard deviation (SD). Analysis of variance (ANOVA) tests were used to determine whether there was a significant difference between the three sample means (neovascular AMD, atrophic AMD, and control). The Student's *t*-test assuming unequal variances was used to determine whether there was a significant difference between the two samples means (neovascular AMD vs. control and atrophic AMD vs. control) to assess the presence of miRNAs that are specific to each type of AMD.

MicroRNA qRT-PCR

For the validation study, the miRCURY LNA Universal RT MicroRNA PCR protocol (Exiqon, QIAGEN Ltd, Manchester, UK) was used to conduct the first-strand cDNA synthesis and qRT-PCR, using the individual assays for human miR-874 and Pick-&-Mix microRNA PCR Panel. The custom serum focus microRNA panel was used for the validation set, which focuses on miRNAs of interest, includes controls, reference genes, RNA spike-in, and interplate calibrators.

Fourteen selected and two endogenous control miRNAs were evaluated from serum in a 96-well plate with all the relevant controls (see Supplementary Table S3 for the panel assay list). In addition, the RNA spike-ins technology ensured the quality of RT and qRT-PCR reactions.[26] A 2 µL RNA was reverse transcribed in 10 µL reactions using the miRCURY LNA Universal RT MicroRNA PCR cDNA synthesis kit (Exiqon). cDNA was diluted 50 times and assayed in 10 µL PCR reactions according to the protocol for miRCURY LNA Universal RT MicroRNA PCR (Exiqon) to determine the C_T value. All PCR reactions for each sample were in triplicate. The qRT-PCR was performed to validate detection of selected miRNAs.

Data Quality Control

The RNA spike-in for quality control of the cDNA synthesis and qPCR experiment was used to ensure that the quality of the input RNA was high enough for effective amplification. RNA spike-in control UniSp6 (CP) was used to evaluate the RT reaction. The expression level of this assay indicates that the reverse transcription was successful; inter-plate calibrators UniSp3 (IPC) evaluates the qPCR reaction, which indicated good technical performance of the experiment.

Normalization

For the present study, normalization of serum miRNAs was performed based on the average of the normalizer assays C_T, which included miR-323-3p and miR-324-3p.

The formula used to calculate the normalized v values is:

$$\begin{aligned}&\text{Normalized } C_T \text{ (d } C_T\text{)}\\ &\quad = \text{assay } C_T \text{ (sample)}\\ &\qquad -\text{average } C_T \text{ (Normalizer assays).}\end{aligned}$$

Statistical Analyses

Statistical analyses was performed in Excel (Microsoft) and the R software for Statistical Computing (R Foundation for Statistical Computing, Vienna, Austria) P values were generated using the Student's *t*-tests, with $P < 0.05$ considered as statistically

significant and ANOVA using R for statistical computing.

Results

Profiling Results of TaqMan miRNAs Arrays Detected in Serum

Unsupervised hierarchical clustering analysis (Supplementary Fig. S3) performed for the discovery study using dChip software (available at http://sites.google.com/site/dchipsoft/home) showed a stronger correlation between differential miRNA expression in neovascular and atrophic patients groups compared with the matched controls. Overall, 157, 207, and 190 miRNAs were detected in control, neovascular, and atrophic samples, respectively. The numbers of miRNAs detected in patients' groups were much higher than in the control group, which suggests the presence of specific miRNAs detected in the diseased group that might be considered as biomarkers for AMD ($P \leq 0.051$) (Supplementary Fig. S4).

Identification of Upregulated and Downregulated miRNAs

For the discovery study, we first made case-versus-control comparisons using all 30 participants (i.e., 10 vs. 10). miRNAs that were upregulated (RQ >1.5) or downregulated (RQ <0.5) were selected for further analysis. The selected miRNAs that met this criterion were compared to identify any miRNAs that were commonly expressed in all specimens. We identified 57 upregulated miRNAs with at least 1.5-fold differential levels at the significance level of $P < 0.05$. A total of 53 miRNAs were detected in the neovascular AMD group, 11 miRNAs detected in the atrophic AMD group, and 7 miRNAs shared by 2 groups (Fig. 1), suggesting a potential difference in circulating miRNAs among atrophic and neovascular types, as well as in the control group.

As shown in Supplementary Tables S4 and S5, among a total of 377 miRNAs profiled by TLDA, there were 53 and 11 upregulated miRNAs detected (with detection $P \leq 0.05$) in the serum of neovascular and atrophic AMD patients, respectively, compared with controls.

miRNAs as Candidate Biomarkers

According to the results, some miRNA candidates were common among the atrophic or neovascular AMD groups (ranging from 80%–90%). A Venn diagram was used to select the miRNAs that were common in >70% of atrophic and neovascular AMD groups. The results were as follows: 53 miRNAs were upregulated in 70% to 90% of neovascular AMD patients; 11 miRNAs were upregulated in 70% to 90% of atrophic AMD patients. Seven upregulated miRNAs were in common between neovascular and atrophic AMD patients, namely miR-195, miR-486-5p, miR-16, miR-374a, miR-32-3p, miR-451, and miR-223 (Fig. 1; Supplementary Tables S4 and S5).

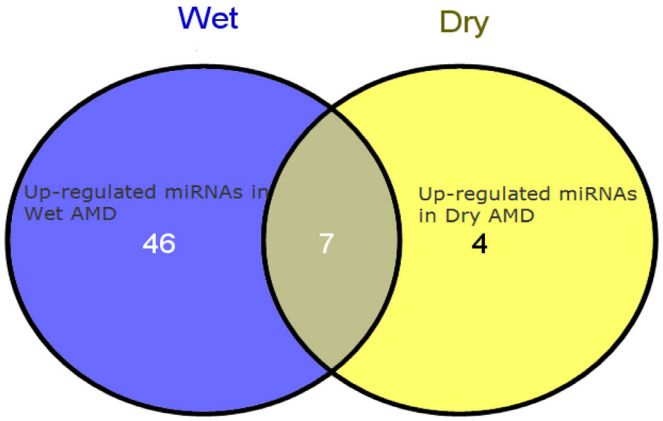

Figure 1. Venn diagram of differentially expressed miRNA, showing the overlap of seven upregulated miRNAs between dry and wet AMD groups.

miRNAs Selected for Further Validation

Fourteen miRNAs were selected for further validation via qRT-PCR. The selection criteria consisted of an RQ ≥1.5 for upregulated miRNA, as only upregulated miRNAs were commonly expressed in all specimens. The selection of upregulated miRNAs was based on common upregulation in atrophic and neovascular AMD. miR-874, miR-132, miR-410, miR-27b, miR-146a, miR-126, and miR-25 were common in the neovascular AMD group, whereas miR-374a, miR-19a, miR-19b, miR-296-5p, and miR-486-5p were common among the atrophic AMD group. Let-7b and miR-16 were common in both neovascular and atrophic AMD groups (Figs. 2–4). Supplementary Tables S6 and S7 illustrate the expression of miRNAs selected for validation. Serum specimens from 30 atrophic AMD patients, 30 neovascular AMD patients, and 30 healthy controls were included in the analysis.

AMD-miRNAs Biomarkers Validation, Data Quality Control, and Normalization

Each RNA sample was successfully analyzed. The steady levels of the assay shown in Supplementary

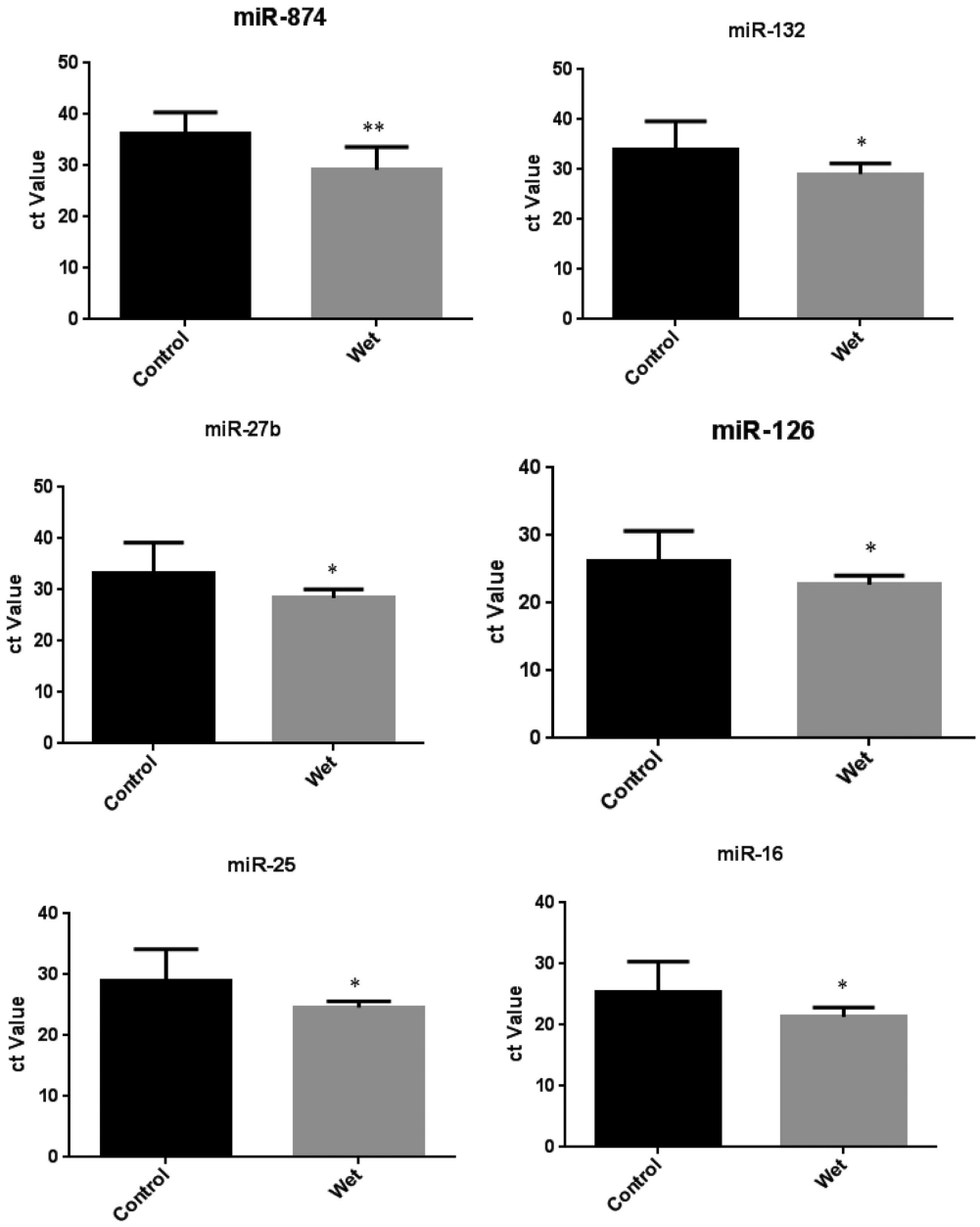

Figure 2. (A) Graphs illustrating the selected miRNAs for neovascular AMD for further validation based on RQ value. Each bar represents the average C_T value ± SD (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$). Wet: neovascular AMD. (B) Graphs illustrating the selected miRNAs for neovascular AMD for further validation based on RQ value. Each bar represents the average C_T value ± SD (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$). Wet: neovascular AMD.

Fig. S5 indicated that both reverse transcription and qRT-PCR were successful.

Normalization was performed based on the average of the assays detected previously in all specimens (miR-323, miR-324). As shown in Supplementary Fig. S6, the raw C_T obtained for the two control miRNA assays were used for normalization (ANOVA; $P = 0.132$). The stability of the average of these two miRNAs is higher than any single miRNA in the dataset as measured by the NormFinder software (available at http://moma.dk/normfinder-software).

Validation of the Pilot Study of the Differentially Expressed miRNAs

The validation panel profiling was successfully completed. These miRNA candidates were common

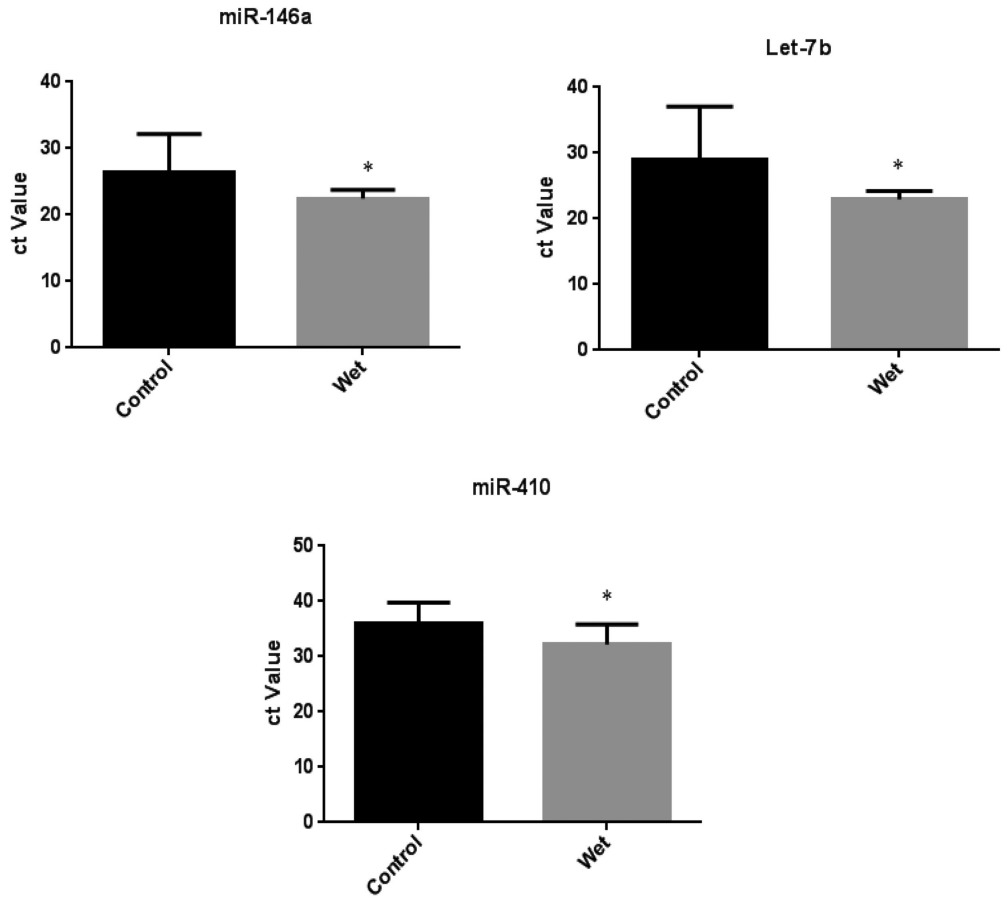

Figure 2. Continued.

among the atrophic or neovascular AMD patient groups, which were chosen as candidate biomarkers of AMD for validation. Supplementary Table S8 shows the individual results for these miRNAs.

miRNAs as Candidate Biomarkers for AMD

Based on the results of the validation study panel, three upregulated miRNAs Table 2; Fig. 5 have been confirmed when comparing the two groups using the Student's *t*-test. miR-126, miR-19a, and miR-410 were found to be at significantly higher levels in AMD specimens versus control.

Discussion

AMD is one of the leading causes of progressive visual impairment in the over-50 population, and eventually leads to irreversible blindness.[27] Traditional

Table 2. miRNA Names, Means, and SD of their RQ Values, Followed by the *P* Values from ANOVA and the Student's *t*-test for the Validation Study[a]

	Control		Atrophic		Neovascular			*t*-test		
Target	Mean	SD	Mean	SD	Mean	SD	ANOVA	Control vs. Neovascular	Control vs. Atrophic	Neovascular vs. Atrophic
miR-126b	2.32	3.23	7.04	6.88	7.02	9.03	**0.011**	**0.011**	**0.00152**	0.994
miR-19a	4.66	6.58	9.28	15	18.18	28.08	**0.021**	**0.015**	0.130	0.133
miR-410	7.61	22.18	25.16	30.72	68.91	152.61	**0.031**	**0.0374**	**0.0142**	0.134

[a]Bold values are statistically significant.

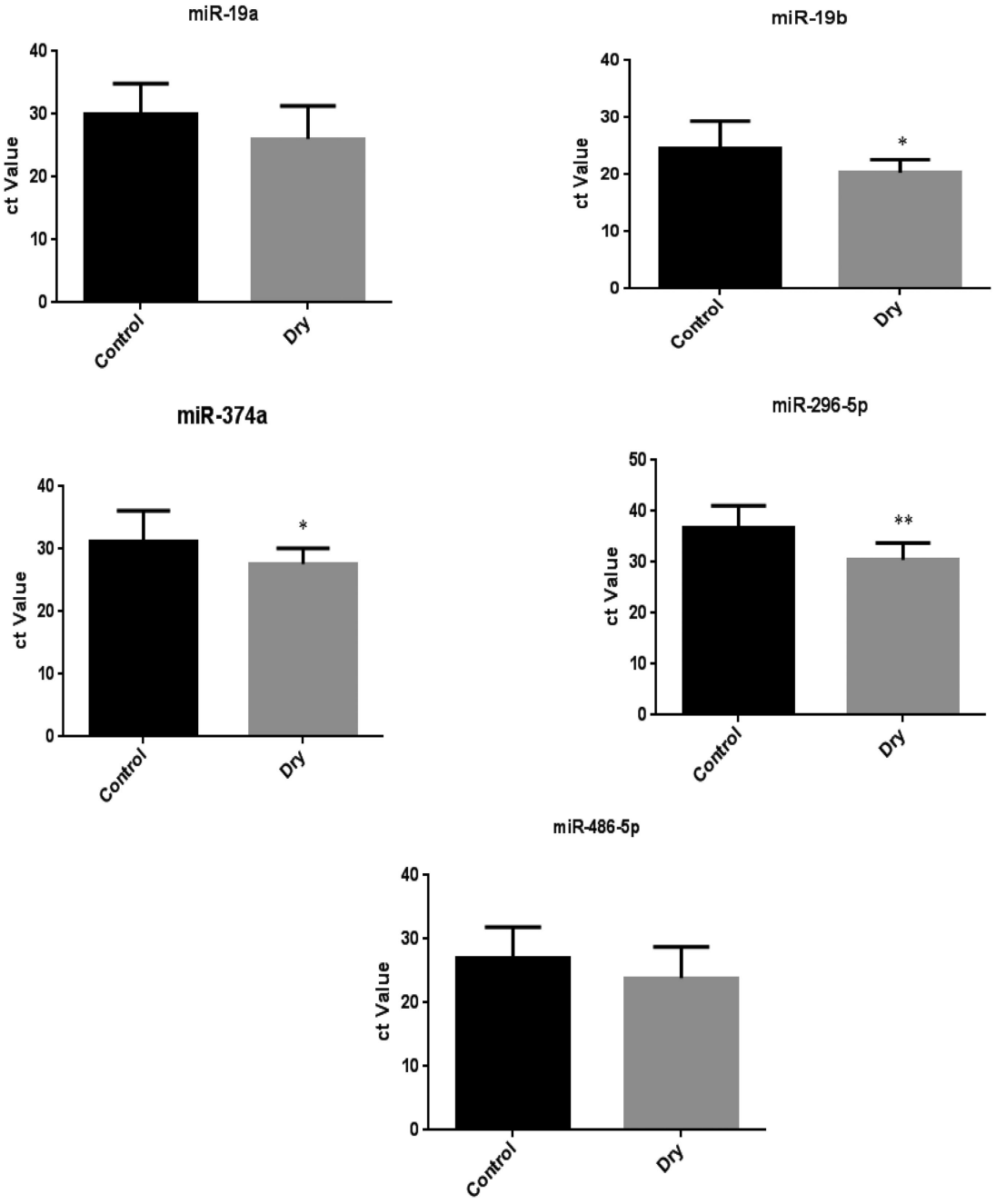

Figure 3. Graphs showing the selected miRNAs for atrophic AMD for further validation based on RQ value. Each bar represents the average C_T value ± SD (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$). Dry: atrophic AMD.

diagnosis by ophthalmoscopy has its limitations as a prognostic screening tool, and identification of blood markers has generally been lacking diagnostically. The lack of simple diagnostic tools available for AMD has motivated us to search for diagnostic biomarkers for AMD. The study by Ren et al.[17] has identified three differently expressed miRNAs in a total of 126 AMD patients, miR-27a-3p, miR-29b-3p, and miR-195-5p, which are considered as novel AMD biomarkers.[22] That study also showed that the level of miR-27a is higher in patients with neovascular (wet) AMD compared with the patients with dry AMD.[17] Moreover, an intensive study profiled miRNAs in the vitreous humor and plasma of patients with neovascular AMD; it demonstrated the potential correlation between vitreal and plasma miRNA signatures with neovascular AMD. The results revealed an increase in miR-146a and a decrease in miR-106b and miR-152 in the vitreous humor, which was reproducible in plasma that emphasizes the potential

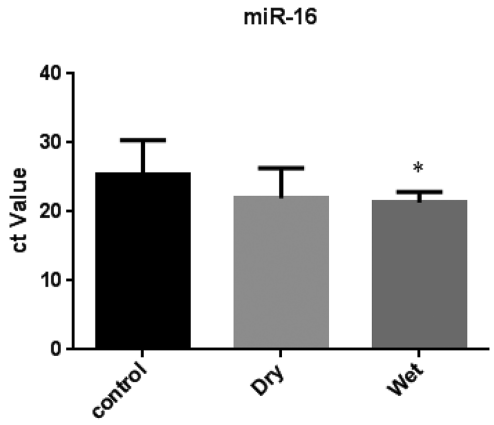

Figure 4. Graph illustrating the selected miRNAs for both AMD groups for further validation based on RQ value. Each bar represents the average C_T value ± SD (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$). P value (ANOVA) = 0.071; neovascular versus control P value = 0.02. Dry: atrophic AMD; Wet: neovascular AMD.

of miRNA biomarkers as diagnostic or prognostic tools.[14]

miRNAs play an important role in gene expression in humans. Overexpression or depletion of individual miRNAs is associated with numerous diseases. They are key regulators of several pathophysiological processes, such as immune and inflammatory responses, pathological angiogenesis, and the response to oxidative stress, all of which have been suggested to be associated with AMD pathogenesis and progression.[1] Dysregulation of miRNAs is involved in retinal diseases, including AMD.[28]

This study has successfully profiled and validated the differential quantities of miRNAs in serum from AMD patients versus controls. The biomarker potential of three miRNAs (miR-126, miR-19a, and miR-410) was confirmed by qRT-PCR, with significantly increased levels in serum from AMD patients compared with healthy controls. The study reveals differences between the atrophic (dry) and neovascular (wet) types, which can act as potential biomarkers of AMD disease. The identification of circulating biomarkers in AMD has undoubtedly shown great promise for their application in a clinical setting.

Vascular endothelial growth factor (VEGF) is a key factor involved in the development of neovascular AMD by enhancing angiogenesis and increasing vascular permeability.[16] miR-126 was described as one of the angiomiRs, which are specific miRNAs that regulate angiogenesis in vivo, and is involved in retinal vascular development or Corneal neovascularization (CNV).[1,29] miR-126, is located within the *egfl7* gene. Deletion of *miR-126* in mice leads to vessel leaking and hemorrhage, this is owing to loss of vascular integrity, and defects in endothelial cell proliferation, migration, and angiogenesis.[1,30] A recent study has suggested that miR-126 inhibits negative regulators of the VEGF pathway. Therefore miR-126 was investigated as a single miRNA in ischemia-induced retinal neovascularization. miR-126 is downregulated in RF/6A cells (endothelial choroidal cells) during hypoxic-induced conditions by regulating angiogenic growth factors.[31] miR-126 negatively regulates VEGF signaling, which can be mediated by different signaling pathways.[30] Furthermore, knockdown of miR-126 in zebrafish leads to loss of vascular integrity and hemorrhage during embryonic development.[32] This implies that miR-126 is interconnected to other miRNAs that are related to angiogenesis and vasculogenesis.[7,13,28]

Chen et al.[33] have shown the underlying mechanism of how miR-410 targets VEGF-A and the potential for treatment of the retinal neovascularization. That study illustrated the mechanism of miR-410 interaction with the 3′-untranslated region (3′-UTR) of the VEGF-A mRNA.

miR-19a belongs to the miR-17∼92 cluster (miRNA cluster encoding miR-17, miR-18a, miR-19a/b, miR-20a, and miR-92a).[33,34] This cluster has an important role in apoptosis and angiogenesis.[35] One of the reports shows that overexpression of the entire miR-17∼92 cluster increased angiogenesis and vascular growth.[36] This suggests the potential proangiogenic effect of miR-19a.[37] Dews et al.[38] also found that miR-19a correlated with decreased expression of several antiangiogenic factors, such as thrombospondin-1 and connective tissue growth factor.[38]

Apoptosis occurs in the RPE cells of patients with AMD, leading to secondary photoreceptor cell death.[39] miR-126 was also shown to regulate cell cycle progression and apoptosis.[40] Additional studies have confirmed that miR-126 could increase the apoptotic effects of irradiation stress in vitro, and deregulation of miR-126 expression inhibited cell proliferation, migration and invasion, and induced apoptosis.[41,42]

miR-19 (a and b) play an important role in regulating cell proliferation, differentiation, and apoptosis.[43] Despite the fact that AMD is a disease associated with aging and the miR-17∼92 cluster is downregulated during aging and senescence,[44] the results presented show that miR-19a was upregulated in AMD patients compared with controls. This suggests both potential AMD biomarker and a treatment target for atrophic AMD.

Dysregulation of the complement system has a role in the pathogenesis of AMD.[45] It has been demonstrated that miR-126 regulates the innate response, and miR-126 has also been found in the blood of patients with systemic lupus erythematosus, which is a

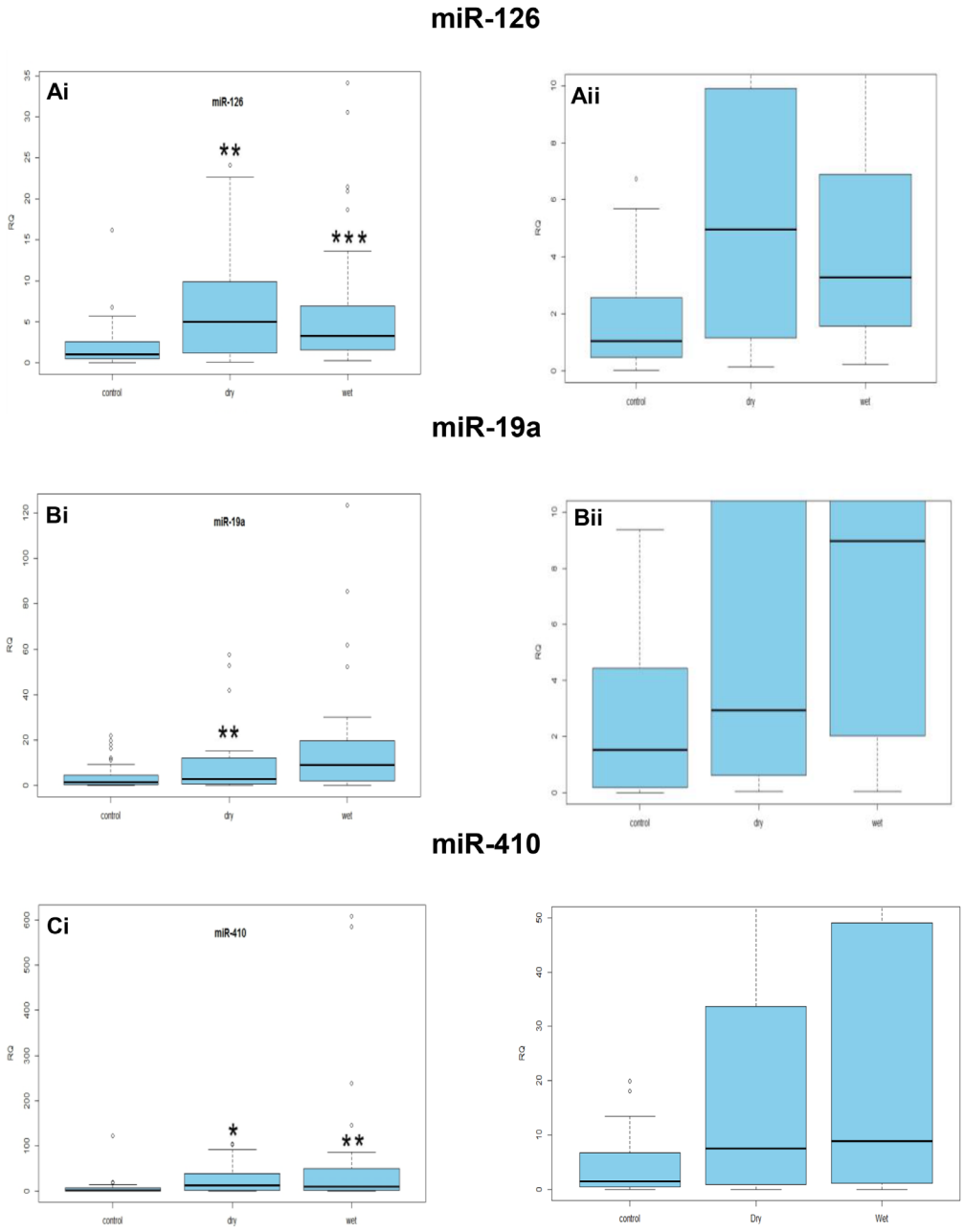

Figure 5. miRNA expression levels (RQ) for three patient groups in the validation study (control = patients free from AMD [and diseases with similar symptoms], atrophic [dry] = patients exhibiting the atrophic form of AMD, neovascular [wet] = patients exhibiting the neovascular form of AMD) for three target miRNAs of interest: Ai,ii: miR-126, Bi,ii: miR-19a, Ci,ii: miR-410, Figure Ai, Bi, Ci: including the data points outliers. Figure Aii, Bii, Cii (enlarged): the boxplot format is as follows: the median value is represented by a dark central bar within each box, the box represents the interquartile range (between the lower and upper quartiles), the whiskers represent the rest of the data, except outliers, which are points that lie more than 1.5 times the interquartile range away from the interquartile boundaries (cut off at RQ = 50). *P* values *≤0.05, **≤0.01, ***≤0.001.

neurodegenerative autoimmune disease.[46] It is reported that C1q downregulates the expression of miR-410, which suggests a potential role of miR-410 in neurodegenerative autoimmune diseases (as AMD is considered by some researchers).[15,41]

Analysis of miRNAs, the major contributing in pathophysiological pathways involved in development of AMD (angiogenesis, apoptosis, and complement dysregulation), hints at a complex plausible role for miR-126, miR-19a, and miR-410.

The profiling of miRNAs biomarkers in serum still remains a huge challenge, as we were faced with several limitations in our study, including small sample sizes and biological variation.[15] This study consists of an

elderly population, and for the control population it was difficult to find individuals at this age group without underlying conditions, such as diabetes and hypertension. In addition, a small cohort of patients was used in the discovery study, so to overcome this obstacle, a larger cohort was investigated in our validation study. Nevertheless, further validation across a large group of AMD patients and controls is required.

Conclusions

From our study it is evident that miR-126, miR-410, and miR-19a have potential as diagnostic AMD biomarkers. It is clear that we still struggle, as a community, to accurately define and classify the heterogeneous phenotypes in AMD. The ongoing work to improve our classification system will aid our study to clearly define the miRNAs profile for each phenotype.[47,48] Larger numbers of each distinct subtype will be required to definitively allocate diagnostic and prognostic significance to each miRNA.

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