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Serum MicroRNAs as Potential Biomarkers of AMD

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Background: Age-related macular degeneration (AMD) is a major cause of blindness worldwide. Circulating microRNAs (miRNAs) in serum have emerged as novel candidate biomarkers for many diseases. The aim of the present study was to identify a serum microRNA (miRNA) expression profile specific for dry and wet forms of AMD.

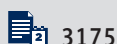
Material/Methods: Serum miRNA expression was first screened using TaqMan® Human MicroRNA Array A (Applied Biosystems). An extensive, self-validated, individual, quantitative RT-PCR (qRT-PCR) study was then performed on a cohort of 300 AMD patients (150 wet form and 150 dry form) and 200 controls. The Mann-Whitney U test and non-parametric Spearman's rank correlation coefficient were used for statistical analysis.

Results: miRNA expression analysis revealed increased expression of miR661 and miR3121 in serum of patients with dry AMD and miR4258, miR889, and Let7 in patients with wet form. Expression of analyzed miRNA was not observed or remained at low level in controls.

Conclusions: Differences in miRNA serum profile exist between patients with wet and dry form of AMD, which indicates miRNAs as potential biomarkers of AMD. Further studies should be performed to confirm its significance in clinical practice.

MeSH Keywords: **Gene Expression Profiling • MicroRNAs • Serum**

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Background

Age-related macular degeneration (AMD), a progressive chronic disease of the central retina, is a significant health problem in aging populations [1], as it is believed to be responsible for the loss of eyesight in one-quarter of the world's population by the time they reach age 90 [2]. Most of the visual loss occurs in the late stages of the disease due to 1 of 2 processes: neovascular, or wet, AMD and atrophic, or dry, AMD [3]. Although 85 to 90% of AMD patients are diagnosed with the less serious dry form, the wet form of the disease, associated with the growth of blood vessels beneath the retina and their subsequent leakage, often results in serious permanent vision loss. However, recent advances have been made in the treatment of wet AMD, such as the use of pegaptanib, ranibizumab, bevacizumab, and aflibercept, antiangiogenic agents which have shown therapeutic promise for wet AMD by targeting choroidal neovascularization [4–6]. Unfortunately, there is currently no proven treatment for dry AMD in the clinical context.

miRNAs are involved in a variety of fundamental cellular processes such as cellular proliferation, development, migration, and apoptosis regulation. These short strands of RNA are excellent indicators and potential regulators of such activities on the cellular level [7]. MicroRNAs are evolutionarily conserved, and are an abundant class of endogenously-expressed, single-stranded RNA molecules 19–25 nucleotides long, which do not code for proteins. They recognize sequences in the 3'-untranslated regions (3'-UTR) of target messenger RNAs, and either induce mRNA degradation [8] or inhibit their translation [9,10]. miRNAs are plentiful in the human genome and exert a range of effects. Between 800 and 1000 types of miRNA are estimated to be present [11], each may have multiple mRNA targets, and up to 30% of human genes are estimated to be regulated to some extent by them [12,13].

miRNAs are also known to influence various aspects of eye development and differentiation [14,15], and thus are regarded as novel therapeutic targets for regenerative eye diseases, as well as cancer and cardiovascular disease [16–18]. Recently, miRNAs have been shown to be directly linked to retinal diseases like retinitis pigmentosa [19], retinoblastoma [20], and ocular neovascularization [21,22]. The miRNAs identified in human serum and plasma are known to be relatively stable, as they have been found to be resistant to RNAase degradation, even in stored samples [23]. This stability has made miRNAs desirable candidates for epidemiological studies, particularly since small serum or plasma samples are needed for miRNA profiling [24,25].

The aim of this study was to identify miRNA profile in patients with dry and wet form of AMD and demonstrate the utility of miRNAs as potential biomarkers of AMD disease. The expression

pattern of various miRNAs in serum was screened using a TaqMan® Human MicroRNA Array A (Applied Biosystems) followed by an extensive self-validated study using individual quantitative RT-PCR (qRT-PCR) assays.

Material and Methods

Sample collection

Sample collection was performed in accordance with a protocol approved by the Committee of Bioethics at the Medical University of Lodz (RNN/228/11/KE), Poland. Informed consent was obtained in accordance with Declaration of Helsinki. Blood samples were obtained from 300 AMD patients: 150 patients with the wet form and another 150 with the dry form. In addition, samples were taken from 200 controls hospitalized in Military Teaching Hospital No. 2 Lodz, Poland and the Sal-Med Medical Centre, Lodz, Poland. All patients were diagnosed with the use of OCT (optical coherent tomography) and FAG (fluorescein angiography). All wet AMD patients demonstrated the early form of wet AMD and were newly diagnosed and qualified for anti-VEGF (ranibizumab) therapy. Serum from those patients was taken before first anti-VEGF injection. Patient characteristics are presented in Table 1. All samples were stored at –80°C after collection. Cell-free plasma was isolated from all blood samples within 2 h of collection using a 2-step protocol (1500 r.p.m. for 10 min, 12 000 r.p.m. for 2 min) to prevent contamination by cellular nucleic acids. Plasma was transferred to a fresh tube, leaving a fixed height of 0.5 cm plasma supernatant above the pellet to avoid disturbing it [26].

Patient inclusion criteria

Written consent was obtained from each patient before enrollment onto the study. Healthy volunteers without ocular abnormality served as controls. No statistically significant differences with regard to sex or age were observed between the group of patients and the healthy controls.

Patient exclusion criteria

Patients were excluded from the study on the basis of the following: a diagnosed acute eye inflammation, chronic inflammation, diabetes mellitus and/or rheumatoid arthritis, a body temperature above 38°C for at least 2 weeks, as well as chronic consumption of alcohol, nicotine or narcotics. In addition, pregnant and lactating women were also excluded from the study on the basis of ethical and legal requirements.

RNA extraction

Total RNA was isolated from 400 µl serum taken from AMD patients and controls using the mirVana PARIS Kit (Ambion)

Table 1. Characteristics of patients with AMD and controls.

		AMD patients		Controls		Total
Residence	Village	69	(23%)	66	(33%)	135
	City	231	(77%)	134	(67%)	365
Financial status	Unsatisfactory	111	(37%)	80	(40%)	191
	Satisfactory	132	(44%)	90	(45%)	222
	Good	57	(19%)	30	(15%)	87
Smoking status	Non-smoking	195	(65%)	120	(60%)	285
	Smoking (≤ 1 ppp)	51	(17%)	50	(25%)	89
	Smoking (≥ 1 ppp)	54	(18%)	30	(15%)	76

ppd – packs per day (cigarette smoking).

according to the manufacturer's protocol. The sample input comprised 3 μ l of the final eluate. Prior to purification, each plasma sample was spiked with 30 pg of ath-miR-159a, a synthetic, non-human miRNA as a positive control to monitor RNA isolation and as positive control for real-time amplification. The concentration and quality of eluted RNA was measured with a Picodrop Microliter UV/Vis Spectrophotometer (Picodrop Ltd). RNA quality was determined with an Agilent RNA 6000 Nano Kit using a 2100 Bioanalyzer (Agilent Technologies). The degradation rate of total RNA was determined using RIN values. Only the samples with RIN >7 were further analyzed. Directly after isolation, RNA was used for the reverse transcription process.

Screening of AMD associated miRNA genes

Megaplex™ RT Primers Human Pool A and B were purchased from Applied Biosystems and used to prepare the reverse transcription reaction according to manufacturer's recommendation. Real-time PCR was performed using a 7900 HT System (Applied Biosystems) using TaqMan® Human MicroRNA Array A and B purchased from Applied Biosystems. The expression levels of 377 human miRNA genes were assessed in AMD patients and controls. miRNA with altered expression profiles were chosen for further investigations.

Reverse transcriptase reaction

TaqMan® MicroRNA Reverse Transcription Kit and the stem-loop RT primers for selected miRNAs were purchased from Applied Biosystems and used to synthesize cDNA according to the guidelines provided by the manufacturer. Briefly, 10 ng of total RNA was added to the reaction tube to make up a final volume of 15 μ l reaction mix, which was then incubated (30 min at 16°C and 30 min at 42°C) in the thermocycler (Biometra)

Real time RT-PCR

QPCR was performed with an Mx3005P qPCR instrument (Stratagene) using Brilliant QPCR Master Mix (Stratagene) and TaqMan® MicroRNA Assays (Applied Biosystems) for selected miRNAs. The reaction was performed at 95°C for 10 min, followed by 40 amplification cycles at 95°C for 15 sec and 60°C for 1 min. One nanogram of cDNA was used in each PCR reaction, and all samples were amplified simultaneously in triplicate in a single run. The U6 small nuclear RNA served as a reference gene. Melting curves were generated for each real-time RT-PCR to verify the specificity of the PCR reaction. All samples were amplified simultaneously in triplicate in a single run. Relative quantification of mRNA was determined by comparative Ct [27]. The miRNA level was calculated as $2^{-\Delta\Delta C_t}$, while relative expression analysis of the examined gene was presented as an n-fold change in gene expression normalized to a reference gene relative to the control.

Detection and quantification of VEGF and VEGFR2 gene expression

All analyses regarding the expression of the VEGF and VEGFR2 genes on the mRNA and protein levels were performed according to methods previously described [28,29].

Statistical analysis

The results were described with use of descriptive statistics of location (median and mean) and variability (standard deviation and range). All calculations were performed using STATISTICA Version 10 software (StatSoft). Data was ln transformed to obtain a normal distribution. The Mann-Whitney U test was used to compare the distributions of the miRNA expression ratios between the wet and dry AMD groups. Associations between

covariates of interest (demographic and clinical), which demonstrated non-normal distributions for most variables, were qualified by the non-parametric Spearman rank correlation coefficient. In all analyses, statistical significance was considered for p -value ≤ 0.05 .

Results

Sample collection

Research group: AMD patients and controls were selected on the basis of age and sex: 62 female and 88 male with the dry form of AMD, 72 female and 78 male with the wet form, and 89 female and 111 male controls. The mean age of the AMD patients was 67.6 ± 5.6 , while the mean age of controls was 67.3 ± 4.1 . No significant differences were observed with regard to sex ($p=0.26$), age ($p=0.49$), differences in residence ($p=0.16$), financial status ($p=0.74$), and smoking status ($p=0.37$). Detailed data are given in Table 1.

Analysis of the miRNA panel in AMD patients

It has been previously reported that some miRNAs are over-expressed in retinitis pigmentosa, retinoblastoma, and ocular neovascularization in biological samples such as the retinal pigment epithelium (RPE). To screen for miRNA associated with AMD in serum, 10 serum samples from patients (5 wet, 5 dry) and 10 controls were analyzed. The expression level of 377 miRNA genes was measured with TaqMan human miRNA arrays. Approximately 20% of analyzed miRNAs revealed alterations in gene expression profile, of which, 23 demonstrated higher and 8 lower expression in AMD patients compared to controls. RQ values (n-fold change expression level) are presented in Table 2. Of the genes whose expression profile differed from controls, the expression of miR-661, miR-3121, miR-4258, miR-889, and Let-7 differed between dry and wet AMD patients, while the expression of miR-424-5p, miR-301-5p and miR-438 did not. These were selected for further investigations.

Expression profile of selected miRNA genes in AMD patients and healthy controls

The RT-QPCR assay revealed that all selected miRNAs were detectable in tested materials, and the difference in miRNA expression between both sets of AMD patients and controls was highly significant ($p < 0.0001$). An analysis of the expression of miR-661, miR-3121, miR-4258, miR-889, and Let-7 on larger groups revealed significant differences between patients with dry and wet AMD (Table 3, Figure 1). The obtained results reveal differences in the occurrence of circulating miRNA in the serum of patients depending on the form of AMD. miR-661 level (4,7x) and miR-3121 (3x) were higher in the dry

than the wet form, while miR-4258 (3,3x), miR-889 (3x), and Let-7 (2,6x) were higher in the wet than the dry form. No statistically significant differences were observed between wet and dry forms with regard to the occurrence of miR-438, miR-424-5p or miR-301-5p.

No significant correlation was observed between demographic data (sex and age) and the expression ratios of miRNAs, neither in dry nor in wet AMD. The Spearman rank correlation coefficients are presented in Table 4.

The relationship between the expression of VEGF and VEGFR2 genes and mRNA and protein level was evaluated in blood from AMD patients and controls. The results are presented in Table 5.

Data are presented as the mean \pm SD. (VEGF2) vascular endothelial growth factor receptor 2 gene; (VEGF) vascular endothelial growth factor gene; (P) level of statistical significance

While no significant relationship was found between VEGF and VEGF2 gene expression at the mRNA or protein level in patients with dry form of AMD, significant differences were found on both levels of expression for the wet form.

The following miRNAs were used to identify any correlation between the expression of the serum miRNA genes and the expression of VEGF or VEGFR 2 genes in AMD patients: miR-661, miR-3121, miR-4258, miR-889, miR-438, and Let-7. In dry AMD patients, significant negative correlations were observed only between the expression of VEGF mRNA and protein, and the expression of the miR-661 ($p=0.03$) and miR-4258 genes ($p=0.02$). In wet AMD patients, a significant positive correlation was found between the expression of VEGF and VEGFR2 with the expression of Let-7 miRNA and protein (VEGF-protein: $p=0.02$, VEGF mRNA: $p=0.01$, VEGFR2-protein: $p=0.01$, VEGFR2- mRNA: $p=0.06$). Spearman rank correlation coefficients are presented in Table 6.

Discussion

miRNAs are assuming increasingly greater significance in research aimed at indicating and isolating molecular markers associated with the pathogenesis and prognosis of many types of illness. An understanding of the expression profile of microRNAs in certain diseases may facilitate faster and more precise diagnosis. Most importantly, knowledge of disorders in expression will presumably represent an indication for choice of therapy in individual patients. MicroRNAs also appear to have a universal character for prognosis. Recently, great attention has been paid to circulating microRNAs in serum and plasma with regard to availability of material for tests and ease of analysis. The analysis of miRNA expression has been attempted in ophthalmology [30].

Table 2. miRNAs demonstrating n-fold change in relative expression rate (RQ) in AMD patients without grouping in patients with dry or wet AMD.

	Mean RQ	RQ range
miRNAs up-regulated		
miR-21	13.35	1.21–251.85
miR-31	22.81	1.33–254.25
miR-132	89.24	1.35–1930.22
miR-146a	17.26	0.84–98.54
miR-150	20.63	1.92–322.10
miR-155	22.21	8.21–62.83
miR-200c	15.33	2.13–114.50
miR-204	25.46	1.00–256.54
miR-206	47.09	4.61–166.51
miR-210	11.72	1.88–214.98
miR-223	8.51	3.47–10.11
miR-296	13.37	3.31–148.37
miR-378	21.63	0.92–336.68
miR-519c	34.21	2.41–4082.88
miR-438	3.30	2.32–4.28
miR-661	7.09	3.01–11.82
miR-889	5.91	1.76–10.31
miR-1224	12.81	4.83–8.62
miR-3121	5.85	1.52–9.98
miR4258	5.95	1.29–10.11
Let-7	4.04	2.29–7.24
miR-424-5p	3.62	2.86–4.45
miR-301-5p	4.02	3.35–5.22
miRNA down-regulated		
miR-23	0.10	0.008–0.64
miR-27a	0.003	0.001–0.008
miR-129-3p	0.05	0.001–1.00
miR-139-3p	0.07	0.001–1.78
miR-486-3p	0.01	0.002–0.10
miR-504	0.01	0.004–0.003
miR-618	0.04	0.000–2.53
miR-758	0.06	0.031–0.09

Table 3. Descriptive statistics for expression ratios (RQ) of miRNAs in patients with dry and wet AMD (P-value of Mann-Whitney test for between-group comparisons).

	Form	Median RQ	RQ range	P-value
miR-661	Dry	9.05	6.35–11.82	<0.0001
	Wet	1.96	0.91–3.01	
miR-3121	Dry	8.70	7.42–9.98	<0.0001
	Wet	2.85	1.52–4.18	
miR-4258	Dry	2.60	1.3–3.92	<0.0001
	Wet	8.55	6.99–10.11	
miR-889	Dry	3.00	1.76–4.24	<0.0001
	Wet	8.90	7.51–10.3	
miR438	Dry	3.30	2.32–4.28	0.65
	Wet	3.30	2.48–4.12	
Let-7	Dry	2.70	2.29–3.11	<0.0001
	Wet	6.74	7.23–6.25	
miR-424-5p	Dry	3.97	3.49–4.45	0.60
	Wet	3.28	2.86–3.73	
miR-301-5p	Dry	4.47	3.72–5.21	0.60
	Wet	3.94	3.35–4.53	

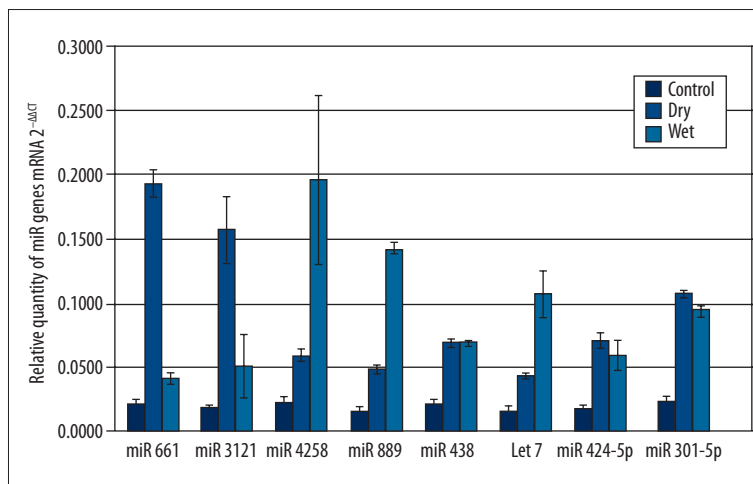


Figure 1. Relative quantity of expression of miRNA genes in serum of AMD patients with dry and wet form vs. controls calculated as $2^{-\Delta\Delta C t}$.

miRNAs have been found to be important regulators of a number of cell functions, including basic maintenance of cell signalling and metabolism. The disruption of these functions in retinal pigment epithelium (RPE) cells can contribute to the development of the chronic atrophy observed in the early phases of AMD. Li et al. showed that changes in gene expression of miR-155 and miR-146a may promote ocular inflammation and proliferation in Graves' ophthalmopathy [31]. Additionally, recent studies have indicated that the following miRNAs are directly associated with the development of AMD and angiogenesis: the miR-17 cluster, miR-27, miR-204, miR-21, miR-132, miR-210, miR-296, miR-378, miR-519c, and the miR-15/107 group [32,33].

AMD is a degenerative and progressive condition involving the RPE, Bruch's membrane, and the choriocapillaries. Despite intensive research, the biochemical and morphological pathogenesis of AMD is still uncertain, due to its multifactorial nature [34]. Many interacting factors (metabolic, genetic, and environmental) seem to have an important influence on the initiation and progression of pathological changes in the macula. Given the clinical and pathophysiological features of AMD, there are 2 forms of this disease. The first, affecting 90% of AMD patients, is the dry form, which is also known as the exudative or atrophy form. Impaired vision in these patients is due to the atrophy of the light-sensitive retinal cells. This

Table 4. Correlation between demographic data (sex and age) and expression ratios of miRNAs in dry and wet AMD.

	miR-661	miR-3121	miR-4258	miR-889	miR-438	Let-7	miR-424-5p	miR-301-5p
Dry								
Age	-0.05	0.12	0.25	0.03	-0.16	-0.17	-0.14	-0.19
Sex	-0.12	-0.20	0.01	0.12	0.11	-0.26	-0.11	-0.21
Wet								
Age	0.06	0.01	-0.10	-0.16	0.07	0.02	-0.11	-0.15
Sex	-0.10	-0.21	0.08	-0.21	-0.28	0.01	-0.13	-0.16

Table 5. The relationship between VEGF and VEGFR2 gene expression on mRNA and protein levels in the wet and dry form of AMD compared to controls.

	Controls	Dry form	Wet form
VEGF mRNA expression 2 ^{-ΔΔC t}	0.23±0.06	0.21±0.06 P=0.001	0.35±0.04 P<0.005
VEGF protein level pg/ml	436.42±60.11	497.76±82.49 P<0.001	751±51.2 P<0.001
VEGFR2 mRNA expression 2 ^{-ΔΔC t}	0.14±0.05	0.20±0.08 P=0.001	0.30±0.05 P<0.001
VEGFR2 protein level pg/ml	121±39.4	108±42.7 P=0.002	226±30.2 P<0.005

Table 6. Correlation coefficients between the expression of VEGF and VEGFR2 genes and miRNA in serum.

	miR-661	miR-3121	miR-4258	miR-889	miR-438	Let-7
Dry AMD						
VEGF_mRNA	-0.30	-0.18	-0.32	-0.03	0.04	0.01
VEGF_protein	-0.29	-0.19	-0.32	-0.06	0.04	0.02
VEGFR2_mRNA	0.03	0.15	-0.03	0.09	-0.01	0.02
VEGFR2_protein	-0.00	0.17	-0.09	0.10	0.02	0.02
Wet AMD						
VEGF_mRNA	-0.06	-0.02	0.04	0.05	-0.19	0.35
VEGF_protein	-0.05	-0.03	0.04	0.02	-0.21	0.33
VEGFR2_mRNA	0.06	-0.02	-0.06	-0.13	-0.08	0.27
VEGFR2_protein	0.01	0.01	-0.04	-0.24	-0.14	0.36

form rarely leads to loss of central vision, but more commonly results in cloudy vision. Dry AMD can develop over a period of many years; patients are obliged to self-control with the help of an Amsler test, regular ophthalmological examinations, and supplementation with multivitamins and antioxidants (AREDS 2001).

Approximately 8–10% of AMD patients develop the wet form of the illness, although some sources indicate this to be as high as 20%. Wet macular degeneration occurs when the retina begins to develop abnormal blood vessels, leading to damage or death of photoreceptors and the retinal pigment epithelial cells which nourish them. The newly formed vessels are twisted, weak, and leaky, which often leads to exudation

and hemorrhage [35]. Therefore, wet AMD has rapid progression and far worse prognosis in comparison with its dry form. In as many as 90% of patients with wet AMD, practical blindness occurs as a result of an increase in abnormal neovascularization of the blood vessels (CNV) during its course. In most cases, the progress of the illness is fast, leading to practical blindness in the affected eye within 2 years. Worsening and loss of eyesight may then occur very quickly, sometimes in the course of a day or a week. Early detection of clinical signs of wet AMD is necessary for effective treatment, such as anti-VEGF therapy. Delays in treatment of more than 28 days are significantly associated with progressive decreases in visual acuity.

Currently, optical coherence tomography and fluorescein angiography can be used for imaging and following the course of AMD; however, any additional non-invasive and sensitive test which can improve the process of diagnosis and assessment of progression of AMD would be important.

The aim of this study was to identify miRNA profile in patients with dry and wet forms of AMD and to demonstrate miRNAs as potential biomarkers of AMD disease. The present study used serum samples obtained from 300 patients who developed the wet or dry form of AMD and analyzed serum miRNAs expression and (iii) compared with the results of 200 controls. All patients with wet AMD were qualified for first anti-VEGF injection.

A set of 31 miRNAs were identified as differentially expressed in the serum of patients with wet and dry forms of AMD, compared to controls. Of these miRNAs, 23 were up-regulated compared to controls, while 8 were down-regulated: miR-661 (4,7x) and miR-3121 (3x) were expressed at significantly higher levels in dry AMD; miR-4258 (3,3x), miR-889 (3x), and Let-7 (2,6x) in wet AMD; and miR-438, miR-424-5p, and miR-301-3p were expressed at similar levels in both wet and dry AMD. These trends were observed in all studied patients with wet and dry AMD.

Endothelial cells are required for angiogenesis from pre-existing vessels. Correlating the gene expression of these serum miRNAs with VEGF and VEGFR2 gene expression in AMD patients would confirm their potential as biomarkers of wet AMD. A significant negative correlation was found between the expression of VEGF on both the protein and mRNA levels, and the expression of miR-661 and miR-4258 in dry AMD patients. Conversely, a positive correlation was identified between the

expression of VEGF and VEGFR2, on both the mRNA and protein levels, and the expression of Let-7 microRNA in wet AMD patients. Let-7 family members are among the most highly expressed miRNAs in retinal tissues and angiogenic endothelial cells; they are known to be pro-angiogenic miRNAs which are positively regulated by the AGO2 (Argonaute 2) that is strongly induced in vascular endothelial cells [36]. Our findings may suggest that Let-7 microRNA plays a role in the neoangiogenesis process in patients with wet AMD.

It has been recently demonstrated that 3 circulating microRNAs are differentially secreted in the serum of wet AMD patients: hsa-mir-361-5p, hsa-mir-301-3p, and hsa-mir-424-5p [37]. The combined profile of these 3 miRNAs was closely associated with the wet form of AMD. To evaluate subtype-specificity, an additional 59 AMD cases with pure unilateral or bilateral geographic atrophy (GA) were analyzed for the hsa-mir-424-5p, hsa-mir-361-5p, and hsa-mir-301-3p microRNAs. No statistically significant differences were found between GA AMD and controls, neither individually nor for a combined microRNA profile; hsa-mir-424-5p levels remained significantly higher in GA AMD when compared to the wet form [37].

The present study also found that the expression of serum miR-424-5p and miR-301-5p was higher in AMD patients than in controls. However, no differences in miR-424-5p and miR-301-5p gene expression were found between wet and dry AMD patients.

Conclusions

Our findings indicate that differences in miRNAs serum profile exist between patients with wet and dry form of AMD. The specific expression of miRNAs for dry and wet AMD may be useful as a biomarker of AMD, but further studies should be performed to confirm its significance in clinical practice. For example, does expression of specific miRNAs for wet AMD occur earlier than its clinical signs, and is miRNAs expression profile correlated with lack of response to antiVEGF2 therapy?

To summaries, this is the first study to compare the expression of genes for miRNAs circulating in the serum of AMD patients. It reveals differences between the dry and wet forms which can act as potential biomarkers of AMD disease.

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