

# Association of *hsa-mir-328-3p* Expression in Whole Blood With Optical Density of Retinal Pigment Epithelial Cells

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**Abstract.** *Aim: To investigate the association of the pair box 6 gene (PAX6) and hsa-miR-328-3p with optical density of macular pigment. Materials and Methods: We evaluated 112 individuals (34 with moderate myopia, eight with high-degree myopia, and 70 healthy individuals). The optical density of macular pigment was measured using single-wavelength reflectometry. DNA and RNA were extracted from whole blood samples. Expression of hsa-miR-328-3p and genotyping of single-nucleotide polymorphism of PAX6 (rs662702) were performed using Applied Biosystems 7900HT real-time polymerase chain reaction system. Optical density of retinal pigment epithelial cells was evaluated using Fundus plus camera. Results: In the group with myopia, with increasing  $\Delta Ct$  hsa-miR-328-3p, the median optical density of the retinal pigment epithelium decreased statistically significantly ( $p < 0.032$ ). No statistically significant association was found between SNP rs662702 genotype variant of the PAX6 gene and the optical density of the retinal pigment epithelium. Conclusion: The increased expression of hsa-miR-328-3p in the blood indicates a decrease in the optical density of the retinal pigment epithelium in those with myopia.*

Myopia is the most common refractive eye disorder in which parallel light rays pass through the eye's optical system to form an image in front of the retina. The cause of this disorder may be an elongated eyeball or excessive lens refractive power (1).

According to histopathological studies, pathological myopia involves excessive and progressive elongation of the globe,

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*Key Words:* *hsa-miR-328-3p, PAX6, myopia, retinal pigment epithelial cell, optical density.*

with consequential thinning of the retina at the posterior pole. *In vivo* measurements of retinal thickness have become possible with the availability of modern imaging technologies, starting investigations of the relationship between myopia and retinal thickness (2-4). Although some earlier *in vivo* imaging studies (5-7) have not found any associations between the retinal thickness and axial length of the eye, in contrast to histopathological findings, the latest studies have demonstrated an inverse relationship between axial length and total average macular thickness, using relatively high scanning resolution and large samples (2-4).

Macular pigment is protective against macular degenerations (8); the highest concentration of macular pigment is found in the central macula, where it functions as a powerful antioxidant (9) and acts as a filter of actinic high-energy (short-wavelength) blue light before it reaches the photoreceptors or the retinal pigment epithelium, thus limiting photo-oxidative damage to retinal cells (10, 11). Studies have shown the association between the optical density of macular pigment and axial length in individuals with myopia. Patients with a high degree of myopia may possibly have macular degenerative changes. Disorders of druse accumulation and retinal pigment epithelial pigmentation can be detected in the retinal macula. A scientific publication has described that the lower the optical density of the retinal macula pigment is, the lower is the protective factor against macular degeneration (8).

Experimental studies with laboratory animals have shown that the pair box 6 gene (*PAX6*) is important in the central nervous system and ocular development, as well as in the lens and retinal differentiation (12); and mutations in this gene may be directly associated with aniridia, cataracts, and high-degree myopia (13, 14). Functional studies have shown that mutations reduce the potential for transcriptional activation of the *PAX6* gene through DNA binding domains (15), which function as regulators in the gene transcript (14).

Bioinformatics methods have found that the rs662702 single nucleotide polymorphism (SNP) of *PAX6* gene

Table I. Characteristics of participants in the *hsa-miR-328-3p* expression study.

| Characteristic          | Myopia (N=42)         |                       | Control (N=70)     | p-Value          |
|-------------------------|-----------------------|-----------------------|--------------------|------------------|
|                         | Moderate              | High                  |                    |                  |
| Age, years              |                       |                       |                    |                  |
| Median (range)          | 27 (18-40)            | 25 (22-40)            | 30 (18-40)         | 0.203            |
| Gender, n (%)           |                       |                       |                    |                  |
| Female                  | 25 (73.5)             | 6 (75.0)              | 42 (60.0)          | 0.138            |
| Male                    | 9 (26.5)              | 2 (25.0)              | 28 (40.0)          |                  |
| Spherical equivalent, D |                       |                       |                    |                  |
| Median OD (min-max.)    | -3.96 (-5.75 - -3.00) | -7.41 (-7.87 - -6.87) | -0.12 (-0.37-0.37) | <b>&lt;0.001</b> |
| Median OS (min-max.)    | -4.05 (-5.94 - -3.00) | -6.76 (-7.62 - -6.12) | 0 (-0.37-0.37)     | <b>&lt;0.001</b> |

N: Number of individuals; OD: right eye; OS: left eye; D: diopters. Statistically significant p-values are shown in bold.

coincides with microRNA-328 (*miR-328*) binding site (12). Studies have shown that the rs662702 risky allele (C) of the *PAX6* gene may interact with *miR-328* and increase the expression of transforming growth factor-β3 in retinal pigment epithelial cells (RPECs) and may also increase the expression of matrix metalloproteinase, which is involved in normal physiological processes of the extracellular matrix by regulating connective tissue remodeling, and can lead to an extension of the eyeball (16). This risky allele has also been found to mediate *miR-328* binding to the 3'-UTR transcript, which causes inhibition of *PAX6* protein translation (17).

The 2-kb *miR-328* promoter region, present in the JASPAR database (18), contains retinoic acid-responsive elements and appears to be able to regulate *miR-328* expression. RPECs treated with different doses of retinoic acid exhibit markedly increased *miR-328* expression and inhibition of *PAX6* gene expression (19).

Previous case-control studies have shown significant differences in *Homo sapiens miR-328-3p* (*hsa-miR-328-3p*) expression between patients with myopia and controls (20). Therefore, it was relevant to examine *in vivo* the associations between *hsa-miR-328-3p* and RPECs, as well as the *PAX6* gene and RPEC optical density. The results may imply their interaction playing a role in changes in the formation of the RPEC layer which are observed in patients with a high degree of myopia.

## Materials and Methods

**Ethics statement.** Permission (number BE-2-48) to undertake the study was obtained from Kaunas Regional Biomedical Research Ethics Committee. The procedure and purpose of the study were explained to participants and their informed consent to participation was obtained.

**Study sample.** Expression of *hsa-miR-328-3p* and *PAX6* genotyping of SNP rs662702 was studied in 112 twins registered at the Twin Center of Lithuanian University of Health Sciences. Based on the available eye refraction results, the first-borns from the twin pairs

Table II. Associations of the pair box 6 (*PAX6*) gene rs662702 genotypes with optical density of the retinal pigment epithelial cell layer (RPEC OD). Data are the median (range).

| Group   | RPEC OD              |               |         |
|---------|----------------------|---------------|---------|
|         | <i>PAX6</i> rs662702 |               |         |
|         | TT                   | CT            | p-Value |
| Control |                      |               |         |
| N       | 43                   | 14            |         |
| OD      | 239 (198-280)        | 239 (214-305) | 0.667   |
| OS      | 243 (202-293)        | 244 (216-302) | 0.583   |
| Myopia  |                      |               |         |
| N       | 19                   | 5             |         |
| OD      | 239 (214-305)        | 247 (202-297) | 0.733   |
| OS      | 243 (222-338)        | 245 (218-297) | 0.791   |
| All     |                      |               |         |
| N       | 62                   | 19            |         |
| OD      | 239 (198-305)        | 247 (202-297) | 0.748   |
| OS      | 243 (202-338)        | 239 (218-297) | 0.769   |

N: Number of individuals; OD: right eye; OS: left eye.

were selected. Two groups were formed: i) Patients with moderate-/high-grade myopia (N=42) and ii) a control group with no refractive disorder (N=70) (Table I). Patients with cardiovascular disease (arrhythmia, atrial fibrillation) or a respiratory disease (asthma, lung cancer, respiratory tract infection), which are associated with changes in *hsa-miR-328-3p* expression, were excluded from the study. No significant differences were found between the patient and control groups with respect to age and gender ( $p>0.05$ ). Values of spherical equivalents for both eyes differed significantly between the myopia and control groups ( $p<0.001$ ).

**Refraction studies.** Refraction studies were performed using an autorefractometer (Accuref-K9001; Shin-Nippon, Tokyo, Japan) with 1% cyclopentolate solution. The mean spherical equivalent (SFE) of the eye refraction was calculated using the standard formula: SFE=sphere + (cylinder/2).

Table III. Association of *Homo sapiens* miR-328-3p expression with optical density of the retinal pigment epithelial cell layer (RPEC OD). Four *hsa-miR-328-3p* miRNA expression groups were clustered on the basis of quartiles of the  $\Delta C_T$  value: Group 1: from  $-12.42$  to  $-9.00$ ; group 2: from  $-8.99$  to  $-5.20$ ; group 3: from  $-5.19$  to  $0.99$ ; group 4: from  $-1$  to  $4.00$ . Data are the median (range).

| Group   | RPEC OD                                  |               |               |               | p-Value      |
|---------|--|---------------|---------------|---------------|--------------|
|         | $\Delta C_T$ <i>hsa-miR-328-3p</i> group |               |               |               |              |
|         | 1  | 2             | 3             | 4             |              |
| Control |  |               |               |               |              |
| N       | 22                                       | 14            | 14            | 20            |              |
| OD      | 251 (206-305)                            | 235 (218-256) | 239 (210-264) | 243 (210-276) | 0.193        |
| OS      | 249 (202-288)                            | 247 (218-293) | 241 (125-293) | 235 (210-272) | 0.583        |
| Myopia  |  |               |               |               |              |
| N       | 10                                       | 13            | 12            | 7             |              |
| OD      | 264 (210-297)                            | 245 (202-264) | 231 (214-243) | 206 (198-218) | <b>0.032</b> |
| OS      | 258 (206-338)                            | 241 (200-264) | 235 (226-239) | 210 (202-214) | <b>0.020</b> |

N: Number of individuals; OD: right eye; OS: left eye. Statistically significant p-values are shown in bold.

Individuals with refractive SFE in at least one eye  $\geq -0.5$  D were assigned to the myopia group. Individuals with refractive error in both eyes with an SFE of between  $-0.49$  and  $0.49$  D were assigned to the control (emmetropia) group.

The degree of myopia was also defined by the intensity of the corrective lens or the optical power that focused the image on the retina. The results of the eye examinations were recorded using standardized forms.

**Genotyping of SNP.** Genotyping kits developed by Applied Biosystems (Waltham, MA, USA) were used for *PAX6* gene SNP study. The assays were performed using the telomere length-polymerase chain reaction method (PAX6 rs662702 C\_898192\_10; HT 7900; Applied Biosystems, Foster City, CA, USA). Data were analyzed using the Applied Biosystems 7500 Real-Time PCR System User Manual.

**miRNA expression.** Blood for miRNA expression assays was collected in special Tempus™ Blood RNA Tubes (ThermoFisher Scientific, Waltham, MA, USA) with stabilizer and frozen at  $-80^\circ\text{C}$  until the beginning of the assays. The assays utilized Taqman® Advanced miRNA Assay primer set with specific miRNA primers to the cDNA (ThermoFisher Scientific), which consisted of two primers for amplifying the miRNA cDNA region of interest and one probe unique to the sequence used to detect the desired miRNA. *Hsa-miR-328-3p* expression results were evaluated using exogenous (*ath-miR-159a*) and endogenous controls (*ath-miR-16*) according to the TaqMan Advanced miRNA Assays (ThermoFisher Scientific) protocol. The  $\Delta$  cycle threshold ( $\Delta C_T$ ) method was used for the relative quantitative analysis of *hsa-miRNA-328-3p* expression in control and myopia groups according to formulae: i)  $\Delta C_T$  (control) =  $C_T$  (target miRNA) -  $C_T$  (reference miRNA); iii)  $\Delta C_T$  (test samples) =  $C_T$  (target miRNA) -  $C_T$  (reference miRNA) (21).

**RPEC optical density measurements.** RPEC optical density was measured using a Fundus plus camera; the measurements were taken at the center of the macula. Macular pigment optical density was measured using single-wavelength reflectometry (Visucam 500; Carl Zeiss Meditec AG, Jena, Germany).

**Analysis of SNP genotyping of and miRNA expression.** The chi-square test or Fisher's bidirectional criterion was used to evaluate the distribution of variants of the *PAX6* gene polymorphisms.

For comparison of *hsa-miR-328-3p* expression between myopia and control groups, the significance level was obtained by Student's *t*-test. To determine the association of the *PAX6* gene with *hsa-miR-328-3p* expression, four miRNA expression groups were clustered on the basis of quartiles of  $\Delta C_T$  *hsa-miR-328-3p* value: Group 1: from  $-12.42$  to  $-9.00$ ; group 2: from  $-8.99$  to  $-5.20$ ; group 3: from  $-5.19$  to  $0.99$ ; group 4: from  $-1$  to  $4.00$ .

## Results

A study of the *PAX6* gene SNP rs662702 and optical density of the RPEC layer in the myopia and control groups showed no significant association (Table II). However, median RPEC optical density values were found to be significantly different by  $\Delta C_T$  *hsa-miR-328-3p* in the myopia group as measured in both eyes (Table III): As the values of  $\Delta C_T$  *hsa-miR-328-3p* increased, the median optical density of the RPEC layer decreased.

No significant associations were found in the distribution of the SNP rs662702 of the *PAX6* gene between all grades of myopia and the control group ( $p > 0.05$ ). However, significant differences between the *PAX6* gene (rs662702) TT and CT genotypes were detected in moderate and high degree myopia and the risky C allele increased the risk of myopia. The distribution of *PAX6* genotypes between myopia and control group is described in our previous studies (20).

## Discussion

Visual signals originate from retinal photoreceptor and RPECs (22). Studies of the interaction between RPE and scleral cells provide a wealth of information on the development of

myopia. Researchers have found that *miR-328* is present in the ocular tissues of mice (22, 23). In studies on RPECs in which *PAX6* gene expression was inhibited, it was observed that significant changes in the proliferation of biomarkers for myopia in RPECs and sclerosis began to occur. Changes observed in expression of transforming growth factor beta 3, matrix metalloproteinase 2 and integrin  $\beta$ 1, which are important in ocular remodeling, might lead to ocular axis changes that would result in myopia (22).

The *PAX6* gene is responsible for retinal differentiation (12), therefore it is thought that individuals with the *PAX6* gene SNP rs662702 with the risky C allele may have different RPEC optical density compared to individuals without this allele.

A study of *miR-328* and *PAX6* gene expression levels in RPE and scleral cells showed that with increasing *miR-328* expression, *PAX6* gene expression decreased in scleral cells but its increase was observed in RPECs (22). Another study was performed with inhibition of *PAX6* gene expression using different concentrations of *miR-328* in RPECs. This study revealed that a decrease in *PAX6* gene expression is dependent on increasing *miR-328* concentration (19).

In our study of the association of *PAX6* SNP rs662702 with RPEC optical density, we found no significant differences between individuals with myopia and controls. Therefore, it would appear that *PAX6* has no association with RPEC layer formation; however, we found that with increasing expression of *hsa-miR-328-3p*, which participates in regulation of *PAX6* gene expression, the median optical density of RPECs decreased in the group with myopia ( $p < 0.05$ ). Of course, it should be noted that *PAX6* genotyping was performed in a small sample and this may have distorted the results, but a significant association of  $\Delta$ CT *hsa-miR-328-3p* with reduced RPEC optical density shows that a higher expression of *hsa-miR-328-3p* through *PAX6* may affect the formation of the RPEC layer, and retinal thickness. Zereid and Osuagwu found regional differences in the thickness of the retinal layer of healthy eyes, which was dependent on central refraction. This is important when interpreting retinal nerve fiber layer thickness in myopia and disease management (24).

Our study may explain the reason for different retinal thickness between myopic and healthy patients through the mechanism of *hsa-miR-328-3p*. Our recommendations would be to perform more *PAX6* genotyping studies and with larger samples.

## Conclusion

The increased expression of *hsa-miR-328-3p* in the blood indicates a decrease in the optical density of RPEC layer in individuals with myopia.

## Conflicts of Interest

Proprietary interests or conflicts: None of the Authors has any proprietary interests or conflicts of interest related to this submission. This submission has not been previously published anywhere, and it is not under consideration for any other publication.

## Authors' Contributions

EK, BB, RL analyzed the data EK, AS, AV wrote the manuscript EK, AS developed the structure for the paper AS, RL made critical revisions All Authors approved the final article

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Received September 3, 2020

Revised December 3, 2020

Accepted December 8, 2020