miRNA Copy Number Variants Confer Susceptibility to Acute Anterior Uveitis With or Without Ankylosing Spondylitis

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Purpose. To investigate the association of microRNA (miRNA) copy number variants (CNVs) with acute anterior uveitis (AAU) with or without ankylosing spondylitis (AS) and to assess underlying disease mechanisms.

Methods. This study included 768 patients with AAU^+AS^+ or AAU^+AS^- and 660 controls from a Chinese Han population. Genotyping of CNVs was performed by TaqMan PCR. The expression of miRNAs, transfection efficiency of miR-9-3, and cytokine production were measured by real-time PCR, flow cytometry, or ELISA.

Results. The frequency of low copy numbers of miR-143, miR-146a, miR-9-3, and miR-205 and of high copy numbers of miR-301a and miR-23a was increased in patients with AAU^+AS^+ ($P = 3.725 \times 10^{-5}$ to 8.033×10^{-9}). Additionally, the frequency of a low copy number of miR-146a and a high copy number of miR-23a and miR-205 was significantly increased in AAU^+AS^- patients ($P = 0.002$–0.001). The frequency of low copy number of miR-205 was increased in AAU^+AS^+ compared with AAU^+AS^- ($P = 0.001$). The mRNA expression of miR-9-3 was significantly decreased in patients with AAU^+AS^+ compared with controls and positively associated with its copy number. Additionally, the production of IL-1β and IL-6 was shown to be regulated by miR-9-3 in human primary retinal pigment epithelial cells.

Conclusions. Low gene copy numbers of miR-143, miR-146a, miR-9-3, miR-205 and high gene copy numbers of miR-301a and miR-23a were associated with susceptibility to AAU^+AS^+. A low copy number of miR-146a and a high copy number of miR-23a and miR-205 were associated with AAU^+AS^-.

Keywords: miRNA, CNVs, autoimmune disease, AAU^+AS^+, AAU^+AS^-

Uveitis is an intraocular inflammation that can cause blindness.[1] Acute anterior uveitis (AAU) is a common disease characterized by eye pain, photophobia, and vision loss.[2] Approximately 20% to 30% of AAU patients also suffer from ankylosing spondylitis (AS).[3,4] Ankylosing spondylitis is an autoimmune disease that is strongly associated with the human leukocyte antigen B27 (HLA-B27).[5,6] HLA-B27$^+$ AAU accounts for approximately 50% of all cases of AAU, of which 25% also develop AS.[7] Although the etiology and pathogenesis of AAU or AS remain unclear, multiple studies[8–12] have shown that genetic aspects play an important role. Interestingly, a gene expression study has shown that many noncoding regions are associated with AS, such as lnc-ZNF354A-1, lnc-LIN54-1, lnc-FRG2C-3, and lnc-USP50-2,[13] suggesting that noncoding genes are also implicated in this disease.

microRNA (miRNA) are noncoding small molecule RNAs with a length of approximately 19 to 25 nucleotides.[14] miRNAs play important roles in various physiological processes in vivo by inhibiting the target mRNA translation process or by affecting the stability of the mRNA.[15–17] Many studies[18–23] have demonstrated that miRNAs play a role in pathogenesis of human autoimmune and autoinflammatory diseases such as Behcet's disease (BD) and AS. Earlier studies[22] have shown that the miR-146a rs2910164 CC genotype and C allele confer risk for BD. Having more than two gene copies of miR-23a, miR-146a, and miR-301a has been shown to be linked to the susceptibility to Vogt-Koyanagi-Harada (VKH) syndrome.[24] Additionally, the miR-23a rs3745453 C allele acts as a risk factor in the pathogenesis of multiple sclerosis.[25] miR-22 has been suggested to play a negative regulatory role in the pathogenesis of rheumatoid arthritis.[26] Moreover, downregulation of miR-143 plays a vital role in the pathogenesis of ulcerative colitis.[27] miR-9 has been shown to significantly inhibit lymphatic endothelium inflammation.[28] Studies in systemic lupus erythematosus (SLE) reveal a role for miR-205 in disease pathogenesis.[29] The examples shown above indicate that single nucleotide polymorphisms (SNPs) or copy number variants (CNVs) in miRNAs may play a role in the pathogenesis of a variety of immune-mediated diseases. However, the identified SNPs only account for a part of the total estimated disease susceptibility factors, suggesting that other variants may also be implicated in these diseases.

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Gene copy number variation belongs to a structural genetic variation that is caused by a genomic rearrangement.[30,31] The copy number of large fragments of the genome are either augmented or reduced, mainly owing to submicroscopic deletions and duplications. With the in-depth study of gene CNVs, a large number of disease-related CNVs have been found.[32–34] However, no reports are available concerning the association of miRNA CNVs with AAU or AS. Earlier studies on the association of miRNA CNVs with autoimmune disease have included miR-146a, miR-205, miR-9-3, miR-301a, miR-22, miR-23a, and miR-143.[24] As autoimmune diseases may share a similar genetic background, this study was designed to investigate the relationship between these miRNAs and the pathogenesis of AAU^+AS^+ or AAU^+AS^-. The results revealed that CNVs of miR-9-3, miR-301a, and miR-143 were associated with AAU^+AS^+, but not with AAU^+AS^-. Copy number variants of miR-146a, miR-23a, and miR-205, on the other hand, were all strongly associated with both AAU^+AS^+ and AAU^+AS^-.

Materials and Methods

Study Population

This study included 768 patients with AAU including 384 patients with AAU^+AS^+, 384 patients with AAU^+AS^-, and 660 unrelated healthy controls from a Chinese Han population. All individuals were recruited from The First Affiliated Hospital of Chongqing Medical University (Chongqing, China). All patients with an unclear diagnosis were excluded from the study. The participants of this case-control study were matched for age, ethnicity, and geography. Diagnosis of AAU was mainly based on clinical manifestations, limited to the iris and ciliary body.[7] The intraocular inflammation was of sudden onset, with a duration of fewer than 3 months and showed recurrent features.[35] Slit lamp examination was performed in all patients to identify the degree of ciliary congestion, keratic precipitates, anterior chamber cells and flare, and changes of the iris. Fundus fluorescence angiography, optical coherence tomography, and ultrasound biomicroscopy were performed in selected patients for differential diagnosis.[36,37] As for AS, all subjects had radiographic imaging and were diagnosed by rheumatologists according to the Modified New York Criteria.[38] All AS patients in our study conformed to the 1984 Modified New York Criteria for AS diagnosis.[39] HLA-B27 testing was performed in 69.1% of the patients. All participants signed a written informed consent voluntarily and all procedures in this study followed the principles of the Declaration of Helsinki.

DNA Extraction and Genotyping

Genomic DNA was extracted from peripheral blood by using the QIAamp DNA Blood Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. The concentration of genomic DNA was measured by NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA), and diluted DNA (10 ng/μL) was stored at −20°C. Detection of gene copy numbers was performed by the TaqMan-based qPCR in 96-well optical plates on a 7500 real-time PCR system by following the manufacturer's protocols (Applied Biosystems, Foster City, CA, USA). Seven FAM-labeled TaqMan assay probes of our study were as follows: miR-9-3, Hs05330221_cn; miR-22, Hs01095539_cn; miR-23a, Hs04021842_cn; miR-143, Hs03578207_cn; miR-146a, Hs06722002_cn; miR-205, Hs07483345_cn; and miR-301a, CXN1ENO. VIC-labeled RNaseP was used as a standard reference assay for the CNVs (Applied Biosystems). Cycling conditions of copy number genotyping were as follows: 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. All samples were measured in triplicate.

Cell Preparation and Culture

Peripheral blood mononuclear cells (PBMCs) were prepared from venous blood by using Ficoll-Hypaque density-gradient centrifugation. Separated PBMCs were cultured in medium consisting of RPMI 1640 complete medium, 10% fetal calf serum, and 100 U/mL penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) at a concentration of 1×10^6 cells/mL. Peripheral blood mononuclear cells were cultured in 24-well plates and stimulated with lipopolysaccharide (LPS) (100 ng/mL; Sigma-Aldrich Corp., St. Louis, MO, USA) at 37°C in 5% CO_2 for 24 hours after which cytokine expression of interleukin (IL)-1β, IL-6, IL-8, IL-10, IL-17, interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and monocyte chemoattractant protein-1 (MCP-1) was measured in culture supernatants.

Human primary retinal pigment epithelial (hRPE) cells were derived from donors of the Chongqing Eye Bank (Chongqing, China). The hRPE cells were isolated by using trypsin digestion in accordance with a classical protocol.[40] The hRPE cells were separated from freshly enucleated bulbi for corneal transplantation of the donor eyes, which followed the tenets of the Declaration of Helsinki. Briefly, the corneoscleral disc, lens, and vitreous were removed sequentially. The remaining eye cup was incised along the longitudinal direction of the optic nerve. Rinsing with Ca^{2+} and Mg^{2+} Dulbecco's PBS allowed prompt separation of the remaining vitreous and neural retina from the hRPE and permitted detachment of the choroid from the sclera. The hRPE cells adhering to Bruch's membrane on the choroidal sheets were washed with Hank's balanced salt solution (GE Healthcare Life Sciences, Waltham, MA, USA) and treated with 0.25% trypsin-EDTA (Invitrogen, Carlsbad, CA, USA) solution. The cells were suspended in medium (complete Dulbecco's modified Eagle's medium [DMEM] to nutrient mixture F12 [DMEM/F12], 1:1; Invitrogen) containing 10% fetal bovine serum (Invitrogen), 100 U/mL penicillin (Beyotime, Shanghai, China), and 100 ng/mL streptomycin (Beyotime), and planted into 25-cm^2 flasks. The cells were incubated in a humidified 5% CO_2 atmosphere at 37°C. The purity of separated hRPE cells was identified by positive immunostaining with RPE65, an antibody specifically expressed by hRPE cells. Cells at passages 2 to 5 were used for this experiment.

ARPE-19 cells were obtained from the American Type Culture Collection. The culture method of ARPE-19 was the same as used for the hRPE cells. Cells at passages 25 to 26 were used for this experiment.

Transfection of miRNA Mimics and Inhibitors in hRPE Cells

The hRPE cells were seeded in 12-well plates at 2×10^5 cells/well and cultured for 24 hours to reach 50% to 70% confluence and then transfected with miR-9-3 mimics or inhibitors (RIBOBIO, Guangzhou, China) by using 1×CP Buffer and CP Reagent (RIBOBIO) and matched with negative control mimics or inhibitors, respectively. The hRPE cells were starved for 24 hours in DMEM/F12 without serum before further treatments. The cells were harvested for RNA analyses. To detect the effect of miR-9-3 on cytokine production by hRPE, the cells were transfected with miR-9-3 mimics and inhibitors for 6 hours and then stimulated with LPS (1 μg/mL; Sigma) for 18 hours. The production of IL-1β, IL-6, and IL-4 was measured in the culture supernatants.

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Flow Cytometry

To estimate miRNA transfection efficiency in hRPE cells, cells were gently washed with PBS twice after transfection with 5Cy3-labeled mimics for 24 hours and directly analyzed by flow cytometry. Flow cytometry was performed on a FACS Aria cytometer (BD Bioscience, San Diego, CA, USA) and the data were processed with FlowJo software (Treestar, Inc., San Carlos, CA, USA).

RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

Total RNA, including miRNA, was extracted from nonstimulated PBMCs and hRPE cells treated by miR-9-3 mimics and inhibitors (RIBOBIO) by using TRIzol reagent (Invitrogen). RNA concentration was assessed by NanoDrop 2000. The levels of miRNAs were measured by qRT-PCR using miDETECT A Track miRNA qRT-PCR Kit (RIBOBIO) and performed on an ABI 7500 System (Applied Biosystems). The primers for miR-9-3, miR-143, miR-301a, miR-205, miR-23a, miR-146a, and U6 small nuclear RNA were obtained from RiboBio. The sequences were designed by RIBOBIO. Analyses of miRNA expression were normalized to the expression of the internal control U6 by using the $2^{-\Delta\Delta CT}$ method.

Immunofluorescence Staining of hRPE Cells

Isolated hRPE cells were planted on coverslips, then cultured in a cell incubator until they reached 70% to 80% confluence. After fixation with 4% paraformaldehyde for 15 minutes at room temperature, cells were washed with PBS for 4 minutes, which was repeated three times. Fixed cells were treated with a concentration of 0.1% Triton X-100 for 4 minutes and then blocked with 10% goat serum for 30 minutes at room temperature. Cells were incubated with anti-RPE65 antibody (1:100; Novus Biologicals, Littleton, CO, USA) at 4°C for 16 hours. Cells were incubated with Alexa Fluor 594 goat anti-mouse IgG secondary antibody (Proteintech Group, Rosemont, IL, USA) for 1 hour at room temperature after washing three times in PBS. After nuclear staining with 4′,6-diamidino-2-phenylindole (DAPI; Life Technology, Carlsbad, CA, USA) at room temperature in the dark for 2 minutes, fluorescent images were obtained with a fluorescence microscope (model DM6000; Leica, Wetzlar, Germany).

Luciferase Reporter Assay

ARPE-19 and hRPE cells were cultured in a 37°C incubator, in a 5% CO_2 atmosphere. During the logarithmic growth phase, ARPE-19 and hRPE cells were seeded in 96-well plates at a concentration of 1.5×10^4 cells per well and incubated at 37°C for 24 hours. miR-9-3 mimics or nontarget control was diluted by OPTI-MEM medium (Thermo Fisher Scientific), and 3′-UTR of the IL-4 dual reporter gene or mutation carrier was diluted with OPTI-MEM medium. OPTI-MEM medium was also used to dilute the Lipofectamine 2000 reagent (Invitrogen). The three reagents were gently mixed for 5 minutes and then left at room temperature for 20 minutes before the mixture was added to the cells. Luciferase substrate was added to the cells 48 hours after transfection. Stopping reagent was added after 10 minutes, and fluorescence values were measured by using the dual luciferase reporter assay system (Glomax multi detection system; Promega, Fitchburg, WI, USA).

Measurement of Cytokines by ELISA

The production of IL-17, IFN-γ, IL-10, IL-8, TNF-α, IL-6, IL-1β, IL-4, and MCP-1 from LPS-stimulated PBMCs and transfected hRPE culture supernatants was detected by using the human Duoset ELISA development kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocols.

Table 1. Epidemiologic Characteristics of Participants Enrolled in the Present Study

Epidemiologic Characteristics	Total	%
AAU	768	100
Mean age, y ± SD	39.4 ± 12.2*	–
Male	478	62.2
Female	290	37.8
HLA-B27	531	69.1†
HLA-B27$^+$	401	75.5
HLA-B27$^-$	130	24.5
AAU$^+$AS$^+$	384	100
Age of AAU$^+$AS$^+$, y ± SD	39.1 ± 11.5*	–
Male	282	73.4
Female	102	26.6
HLA-B27	288	75.0†
HLA-B27$^+$	265	92.0
HLA-B27$^-$	23	8.0
AAU$^+$AS$^-$	384	100
Age of AAU$^+$AS$^-$, y ± SD	39.6 ± 12.9*	–
Male	196	51.0
Female	188	49.0
HLA-B27	243	63.3†
HLA-B27$^+$	136	56.0
HLA-B27$^-$	107	44.0
Control	660	100
Mean age, y ± SD	39.5 ± 10.4*	–
Male	374	56.7
Female	286	43.3

* The ages indicated are the ages at enrollment.

† The percentages are the proportion of HLA-B27 in AAU, AAU$^+$AS$^+$, and AAU$^+$AS$^-$, respectively.

Statistical Analysis

The differences in copy numbers of miRNAs between patients and controls were analyzed by the χ^2 test using SPSS (v. 17.0; SPSS, Inc., Chicago, IL, USA). The expression of miRNAs and various cytokines was analyzed by the independent samples *t*-test or two independent samples nonparametric test by using SPSS 17.0 software. Bonferroni correction was applied to correct for multiple comparisons. *P* values of CNV comparisons less than $0.05/21 = 0.0024$ and a corrected *P* value of miRNA expression less than 0.05 were considered as significant.

Results

Epidemiologic Characteristics of Patients With AAU$^+$AS$^+$ or AAU$^+$AS$^-$

The details of the epidemiologic characteristics of patients and healthy controls are summarized in Table 1.

Association of miRNA Copy Number Variants With AAU$^+$AS$^+$ or AAU$^+$AS$^-$

A total of 768 patients with AAU (384 patients with AAU$^+$AS$^+$, 384 patients with AAU$^+$AS$^-$) and 660 unrelated healthy controls were recruited for the present study. Subjects enrolled in this

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Table 2. Comparison of Gene Copy Numbers of miR-22, miR-143, miR-205, miR-9-3, miR-146a, miR-23a, and miR-301a in AAU$^+$AS$^+$ and AAU$^+$AS$^-$ Patients

Genes	CNVs	AAU$^+$AS$^-$, Frequency (%)	AAU$^+$AS$^+$, Frequency (%)	Controls, Frequency (%)	P* (AAU$^+$AS$^-$: Controls)	OR (95% CI)	P* (AAU$^+$AS$^+$: Controls)	OR (95% CI)
miR-22	<2	8 (2.1)	13 (3.4)	7 (1.1)	0.170	2.0 (0.7~5.6)	0.008	3.3 (1.3~8.3)
	=2	351 (92.9)	344 (89.8)	620 (93.9)	0.495	0.8 (0.5~1.4)	0.015	0.6 (0.4~0.9)
	>2	19 (5.0)	26 (6.8)	33 (5.0)	0.985	1.0 (0.6~1.8)	0.228	1.4 (0.8~2.4)
miR-143	<2	5 (1.3)	18 (4.8)	1 (0.2)	0.027	8.8 (1.0~75.7)	**1.126×10^{-7}**	**33.2 (4.4~249.9)**
	=2	363 (95.8)	345 (92.0)	647 (98.0)	0.034	0.5 (0.2~1.0)	2.967×10^{-6}	0.2 (0.1~0.4)
	>2	11 (2.9)	12 (3.2)	12 (1.8)	0.253	1.6 (0.7~3.7)	0.156	1.8 (0.8~4.0)
miR-205	<2	3 (0.8)	17 (4.6)	3 (0.5)	0.674	1.7 (0.4~8.7)	**7.187×10^{-6}**	**10.6 (3.1~36.4)**
	=2	362 (95.5)	340 (92.4)	650 (98.6)	0.002	0.3 (0.1~0.7)	2.635×10^{-7}	0.2 (0.1~0.4)
	>2	14 (3.7)	11 (3.0)	6 (0.9)	**0.002**	**4.2 (1.6~11.0)**	0.012	3.4 (1.2~9.1)
miR-9-3	<2	27 (7.2)	43 (11.3)	29 (4.4)	0.059	1.7 (1.0~2.9)	**2.566×10^{-5}**	**2.8 (1.7~4.5)**
	=2	259 (68.5)	222 (58.1)	434 (65.8)	0.364	1.1 (0.9~1.5)	0.014	0.7 (0.6~0.9)
	>2	92 (24.3)	117 (30.6)	197 (29.8)	0.057	0.8 (0.6~1.0)	0.792	1.0 (0.8~1.4)
miR-23a	<2	10 (2.6)	9 (2.3)	0 (0.0)	–	–	–	–
	=2	345 (90.3)	326 (85.6)	640 (97.0)	5.297×10^{-6}	0.3 (0.2~0.5)	7.097×10^{-12}	0.2 (0.1~0.3)
	>2	27 (7.1)	46 (12.1)	20 (3.0)	**0.002**	**2.4 (1.3~4.4)**	**8.033×10^{-9}**	**4.4 (2.6~7.6)**
miR-146a	<2	12 (3.2)	23 (6.1)	3 (0.4)	**0.001**	**7.2 (2.0~25.8)**	**3.716×10^{-8}**	**14.3 (4.3~47.9)**
	=2	361 (96.0)	348 (92.6)	656 (99.4)	1.618×10^{-4}	0.1 (0.04~0.4)	1.732×10^{-9}	0.1 (0.03~0.2)
	>2	3 (0.8)	5 (1.3)	1 (0.2)	0.139	5.3 (0.5~51.1)	0.026	8.9 (1.0~76.3)
miR-301a	<2	7 (1.8)	10 (2.6)	9 (1.4)	0.539	1.4 (0.5~3.7)	0.147	1.9 (0.8~4.8)
	=2	365 (96.6)	354 (92.4)	645 (97.7)	0.264	0.7 (0.3~1.4)	4.061×10^{-5}	0.3 (0.2~0.5)
	>2	6 (1.6)	19 (5.0)	6 (0.9)	0.325	1.8 (0.6~5.5)	**3.725×10^{-5}**	**5.7 (2.3~14.4)**

Bold values indicate statistical significance.

* Bonferroni correction for the number of CNVs tested by the conditional analysis, P value less than $0.05/21 = 0.0024$ was considered significant. Discrepancy between numbers of individuals is due to missing genotyping data.

study were divided into three groups (having <2, 2, or >2 gene copy numbers) based on the general consideration that the most common count of an miRNA gene copy number is 2. Having more than two copies for miR-23a and fewer than two copies for miR-146a was consistently linked to the susceptibility to both AAU$^+$AS$^-$ and AAU$^+$AS$^+$ (AAU$^+$AS$^-$: miR-23a: $P_{cnv>2} = 0.002$, odds ratio [OR] = 2.4; miR-146a: $P_{cnv<2} = 0.001$, OR = 7.2; AAU$^+$AS$^+$: miR-23a: $P_{cnv>2} = 8.033 \times 10^{-9}$, OR = 4.4; miR-146a: $P_{cnv<2} = 3.716 \times 10^{-8}$, OR = 14.3) (Table 2). For miR-205, an increased frequency of more than two copies was found in AAU$^+$AS$^-$ patients ($P_{cnv>2} = 0.002$, OR = 4.2), while having fewer than two copies had a strong association with AAU$^+$AS$^+$ ($P_{cnv<2} = 7.187 \times 10^{-6}$, OR = 10.6) (Table 2). The frequency of having a low copy number of miR-143 and miR-9-3 and a high copy number of miR-301a was significantly increased in AAU$^+$AS$^+$ compared with healthy controls (miR-143: $P_{cnv<2} = 1.126 \times 10^{-7}$, OR = 33.2; miR-9-3: $P_{cnv<2} = 2.566 \times 10^{-5}$, OR = 2.8; miR-301a: $P_{cnv>2} = 3.725 \times 10^{-5}$, OR = 5.7) (Table 2). Additionally, the frequency of having a low copy number of miR-205 was increased in patients with AAU$^+$AS$^+$ compared with AAU$^+$AS$^-$ ($P = 0.001$, OR = 6.1) (Table 3).

HLA-B27 testing was performed in 288 AAU$^+$AS$^+$ and 243 AAU$^+$AS$^-$ patients. Not all patients underwent HLA-B27 testing owing to logistic problems including costs of the test and because it is not yet included as a diagnostic criterion. To investigate a possible role of HLA-B27 in the association with miRNA copy numbers, we performed a preliminary stratified analysis. The results indicated that miR-143, miR-9-3, miR-23a, miR-146a, and miR-205 CNVs showed a consistent association with AAU$^+$AS$^+$ in HLA-B27$^+$ patients, whereas only miR-205 CNVs showed an association in the HLA-B27$^-$group (HLA-B27$^+$: miR-143: $P_{cnv<2} = 1.865 \times 10^{-8}$, OR = 42.3; miR-9-3: $P_{cnv<2} = 0.002$, OR = 2.4; miR-23a: $P_{cnv>2} = 1.411 \times 10^{-10}$, OR = 5.4; miR-146a: $P_{cnv<2} = 5.626 \times 10^{-10}$, OR = 19.8; miR-205: $P_{cnv<2} = 1.543 \times 10^{-5}$, OR = 11.4. HLA-B27$^-$: miR-205: $P_{cnv<2} = 4.799$

Table 3. The Association of Copy Number Variants of miR-22, miR-143, miR-205, miR-9-3, miR-146a, miR-23a, miR-301a With AAU$^+$AS$^+$ and AAU$^+$AS$^-$ Patients

Genes	CNVs	AAU$^+$AS$^+$, Frequency (%)	AAU$^+$AS$^-$, Frequency (%)	P*	OR (95% CI)
miR-22	<2	13 (3.4)	8 (2.1)	0.282	1.6 (0.7~4.0)
	=2	344 (89.8)	351 (92.9)	0.136	0.7 (0.4~1.1)
	>2	26 (6.8)	19 (5.0)	0.303	1.4 (0.7~2.5)
miR-143	<2	18 (4.8)	5 (1.3)	0.006	3.8 (1.4~10.3)
	=2	345 (92.0)	363 (95.8)	0.030	0.5 (0.3~0.9)
	>2	12 (3.2)	11 (2.9)	0.812	1.1 (0.5~2.5)
miR-205	<2	17 (4.6)	3 (0.8)	**0.001**	**6.1 (1.8~20.9)**
	=2	340 (92.4)	362 (95.5)	0.073	0.6 (0.3~1.1)
	>2	11 (3.0)	14 (3.7)	0.592	0.8 (0.4~1.8)
miR-9-3	<2	43 (11.3)	27 (7.2)	0.050	1.6 (1.0~2.7)
	=2	222 (58.1)	259 (68.5)	0.003	0.6 (0.5~0.9)
	>2	117 (30.6)	92 (24.3)	0.052	1.4 (1.0~1.9)
miR-23a	<2	9 (2.3)	10 (2.6)	0.821	0.9 (0.4~2.2)
	=2	326 (85.6)	345 (90.3)	0.044	0.6 (0.4~1.0)
	>2	46 (12.1)	27 (7.1)	0.019	1.8 (1.1~3.0)
miR-146a	<2	23 (6.1)	12 (3.2)	0.057	2.0 (1.0~4.0)
	=2	348 (92.6)	361 (96.0)	0.041	0.5 (0.3~1.0)
	>2	5 (1.3)	3 (0.8)	0.725	1.7 (0.4~7.1)
miR-301a	<2	10 (2.6)	7 (1.8)	0.479	1.4 (0.5~3.8)
	=2	354 (92.4)	365 (96.6)	0.013	0.4 (0.2~0.9)
	>2	19 (5.0)	6 (1.6)	0.009	3.2 (1.3~8.2)

Bold values indicate statistical significance.

* Bonferroni correction for the number of CNVs tested by the conditional analysis, P value less than $0.05/21 = 0.0024$ was considered significant. Discrepancy between numbers of individuals is due to missing genotyping data.

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TABLE 4. Comparison of Gene Copy Numbers of miR-22, miR-143, miR-205, miR-9-3, miR-146a, miR-23a, and miR-301a in HLA-B27$^+$-AAU$^+$AS$^+$ and HLA-B27$^-$-AAU$^+$AS$^+$

Genes	CNVs	HLA-B27$^+$, Frequency (%)	HLA-B27$^-$, Frequency (%)	Controls, Frequency (%)	P^* (HLA-B27$^+$: Controls)	OR (95% CI)	P^* (HLA-B27$^-$: Controls)	OR (95% CI)
miR-22	<2	10 (3.8)	0 (0.0)	7 (1.1)	0.005	3.7 (1.4~9.7)	–	–
	=2	236 (89.0)	21 (91.3)	620 (93.9)	0.011	0.5 (0.3~0.9)	0.646	0.7 (0.2~3.0)
	>2	19 (7.2)	2 (8.7)	33 (5.0)	0.195	1.5 (0.8~2.6)	0.332	1.8 (0.4~8.0)
miR-143	<2	16 (6.0)	1 (4.3)	1 (0.2)	**1.865×10^{-8}**	**42.3 (5.6~321.0)**	0.066	30.0 (1.8~494.6)
	=2	240 (90.6)	20 (87.0)	647 (98.0)	2.325×10^{-7}	0.2 (0.1~0.4)	0.014	0.1 (0.04~0.5)
	>2	9 (3.4)	2 (8.7)	12 (1.8)	0.145	1.9 (0.8~4.6)	0.077	5.1 (1.1~24.4)
miR-205	<2	13 (4.9)	3 (14.3)	3 (0.5)	**1.543×10^{-5}**	**11.4 (3.2~40.2)**	**4.799×10^{-4}**	**36.4 (6.9~193.1)**
	=2	243 (92.4)	18 (85.7)	650 (98.6)	9.545×10^{-7}	0.2 (0.1~0.4)	0.005	0.1 (0.02~0.3)
	>2	7 (2.7)	0 (0.0)	6 (0.9)	0.042	3.0 (1.0~8.9)	–	–
miR-9-3	<2	26 (9.8)	2 (9.1)	29 (4.4)	**0.002**	**2.4 (1.4~4.1)**	0.264	2.2 (0.5~9.8)
	=2	162 (61.1)	13 (59.1)	434 (65.8)	0.184	0.8 (0.6~1.1)	0.517	0.8 (0.3~1.8)
	>2	77 (29.1)	7 (31.8)	197 (29.8)	0.812	1.0 (0.7~1.3)	0.843	1.1 (0.4~2.7)
miR-23a	<2	8 (3.0)	0 (0.0)	0 (0.0)	–	–	–	–
	=2	219 (82.7)	21 (91.3)	640 (97.0)	1.948×10^{-14}	0.1 (0.1~0.3)	0.167	0.3 (0.1~1.5)
	>2	38 (14.3)	2 (8.7)	20 (3.0)	**1.411×10^{-10}**	**5.4 (3.1~9.4)**	0.167	3.0 (0.7~13.9)
miR-146a	<2	22 (8.3)	0 (0.0)	3 (0.4)	**5.626×10^{-10}**	**19.8 (5.9~66.8)**	–	–
	=2	241 (90.9)	18 (94.7)	656 (99.4)	2.692×10^{-10}	0.1 (0.02~0.2)	0.133	0.1 (0.01~1.0)
	>2	2 (0.8)	1 (5.3)	1 (0.2)	0.199	5.0 (0.5~55.5)	0.055	36.6 (2.2~608.8)
miR-301a	<2	4 (1.5)	0 (0.0)	9 (1.4)	1.000	1.1 (0.3~3.6)	–	–
	=2	254 (95.8)	20 (95.2)	645 (97.7)	0.118	0.5 (0.2~1.2)	0.398	0.5 (0.1~3.7)
	>2	7 (2.7)	1 (4.8)	6 (0.9)	0.043	3.0 (1.0~8.9)	0.198	5.5 (0.6~47.4)

Bold values indicate statistical significance.

* Bonferroni correction for the number of CNVs tested by the conditional analysis, P value less than $0.05/21 = 0.0024$ was considered significant. Discrepancy between numbers of individuals is due to missing genotyping data.

$\times 10^{-4}$, OR = 36.4) (Table 4). In the AAU$^+$AS$^-$ group both HLA-B27$^+$ and HLA-B27$^-$ patients showed a similar association with miR-23a CNVs but not with the other miRNA species tested (HLA-B27$^+$: $P_{cnv=2} = 1.364 \times 10^{-4}$, OR = 0.3; HLA-B27$^-$: $P_{cnv=2} = 0.002$, OR = 0.3) (Supplementary Table S1).

The Expression of miRNAs in AAU$^+$AS$^+$ or AAU$^+$AS$^-$

As there was a strong correlation between CNVs of miR-143, miR-205, miR-301a, miR-9-3, miR-23a, miR-146a and AAU$^+$AS$^+$ or AAU$^+$AS$^-$, the expression of these miRNAs in PBMCs was investigated. All patients included in the study showed active disease and were not yet treated with any immunosuppressive drugs for at least 1 month before entering the study. The mRNA expression of miR-9-3 and miR-143 in PBMCs was significantly decreased in patients with AAU$^+$AS$^+$ or AAU$^+$AS$^-$ compared to healthy controls (miR-9-3: $P_{(AAU^+AS^-)} = 0.014$, $P_{(AAU^+AS^+)} = 0.013$; miR-143: $P_{(AAU^+AS^-)} = 0.003$, $P_{(AAU^+AS^+)} = 0.006$) (Fig. 1). However, a significantly decreased expression of miR-146a was found in AAU$^+$AS$^+$ patients compared to healthy controls or to AAU$^+$AS$^-$ ($P = 0.010$, $P = 0.012$) (Fig. 1). There was no significant association of the expression of miR-301a, miR-205, and miR-23a between patients and controls (Fig. 1).

The Influence of Copy Number Variation in miR-9-3 on the mRNA Levels in PBMCs

Peripheral blood mononuclear cells were obtained from healthy controls and used for experiments to study the effect of miR-9-3 CNVs on cytokine production. We used healthy controls since most of our patients were receiving anti-inflammatory treatment, which might affect the results, and sample size of various CNV groups in untreated patients would therefore be too low to produce meaningful results. As the frequency of fewer than two copies for miR-143 and miR-146a was very low in controls (0.2%; 0.4%), while the frequency of fewer than two copies for miR-9-3 was relatively high in controls (4.4%), further studies were performed to estimate the mRNA expression of miR-9-3 in relation to the gene copy numbers. The result showed a significantly increased mRNA expression of miR-9-3 in the high copy number group (CNV > 2) as compared with the low copy number group (CNV < 2) ($P = 4.355 \times 10^{-4}$) (Fig. 2).

The Influence of miR-9-3 CNVs on Cytokine Production in PBMCs

Since the different genotypes of miR-9-3 had an effect on miR-9-3 mRNA expression, we performed a further experiment to measure whether different CNVs of miR-9-3 influenced cytokine production. Culture supernatants of LPS-treated PBMCs from genotyped healthy controls were tested for IL-1β, IL-6, IL-8, IL-10, IL-17, IFN-γ, TNF-α, and MCP-1. A significantly decreased production of IL-1β and IL-6 was found in individuals with low copy numbers of miR-9-3 compared to the high-copy-number group (IL-1β: $P = 0.005$; IL-6: $P = 0.045$) (Fig. 3). There was no significant effect on the secretion levels of IL-8, IL-10, IL-17, IFN-γ, TNF-α, and MCP-1 (Fig. 3).

Identification of Cultured hRPE and ARPE-19 Cells With RPE65

The bovine RPE65 microsomal membrane protein was used as a retinal pigment epithelium marker to identify purity of isolated hRPE and ARPE-19 cells. RPE65 was located in cytoplasmic, microsomal, and cell membrane around the DAPI-positive nucleus of primary hRPE cells (Supplementary Fig. S1). This shows that the primary hRPE cells were RPE-derived cells. The labeled cells were counted with five nonrepeating regions with a fluorescence microscope. The number of positive cells with both RPE65 and DAPI was calculated. The positive rate was 96.6% ± 2.4% in hRPE, and 94.4% ± 5.5% in ARPE-19 cells (Supplementary Fig. S1).

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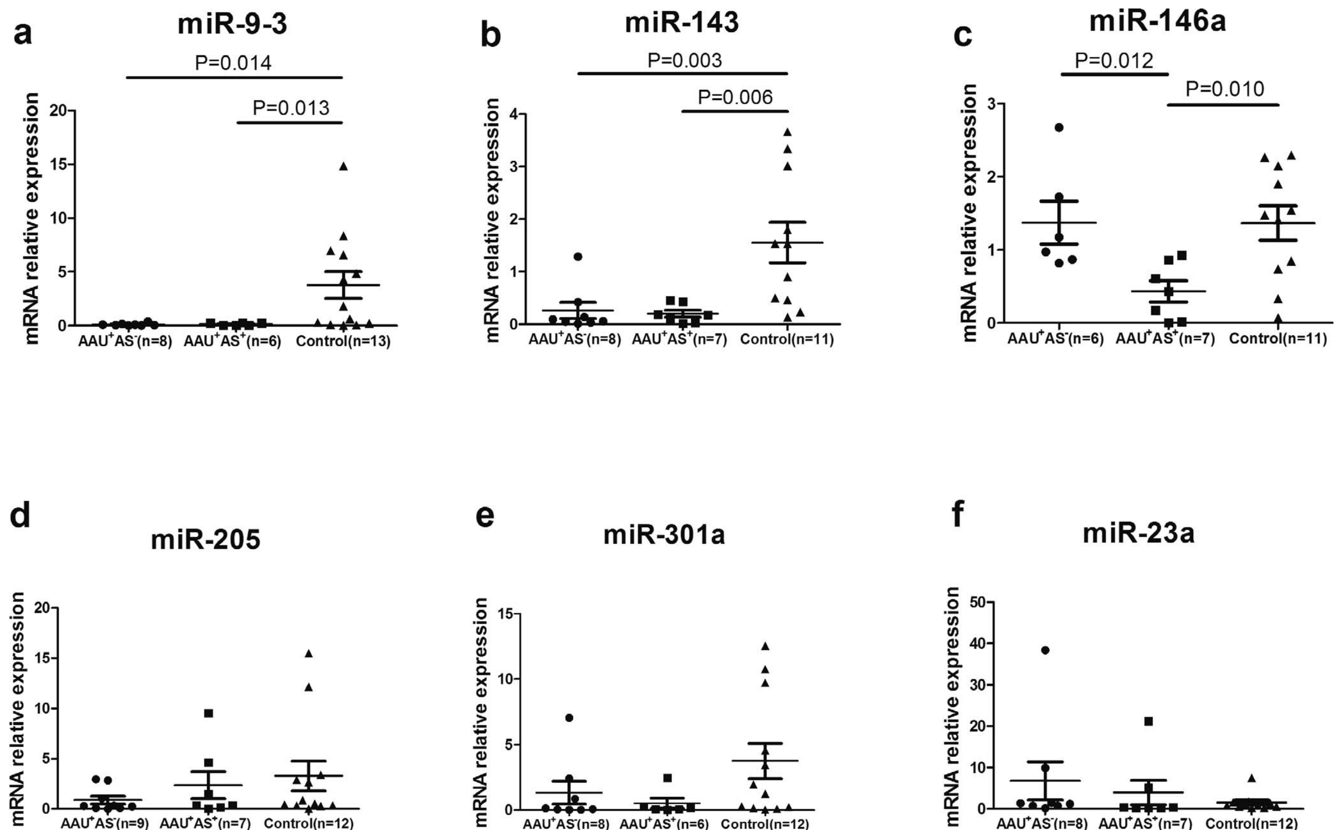

FIGURE 1. The mRNA expression of miRNAs in PBMCs of AAU^{+}AS^{+} or AAU^{+}AS^{-} patients and normal controls. The mRNA expression of miR-9-3 (**a**) and miR-143 (**b**) was significantly decreased in AAU^{+}AS^{+} or AAU^{+}AS^{-} patients compared to healthy controls. A significantly decreased expression of miR-146a was found in AAU^{+}AS^{+} patients compared to healthy controls. Significance was tested by two independent samples nonparametric test or 1-way ANOVA test.

Transfection of miR-9-3 Mimics and Inhibitors in hRPE Cells

Flow cytometry was used to detect the transfection efficiency of 5Cy3-labeled miRNA mimic control. More than 81% of the cells showed a successful transfection of hRPE cells (Supplementary Fig. S2). In accordance with the recommendations of the reagent supplier's manual, concentrations of 50 nM and 100 nM for miR-9-3 mimics, and 100 nM and 200 nM for miR-9-3 inhibitors, were chosen for the further study. The mRNA expression of miR-9-3 was measured by qRT-PCR to obtain the optimal concentration of transfection. For both the miR-9-3 mimics and inhibitors, the lower concentration showed the same effect of an optimal overexpression and inhibition as the high concentration. Therefore, concentrations of 50 nM and 100 nM for miR-9-3 mimics and inhibitors, respectively, were chosen to transfect the hRPE cells in further experiments (Supplementary Fig. S3).

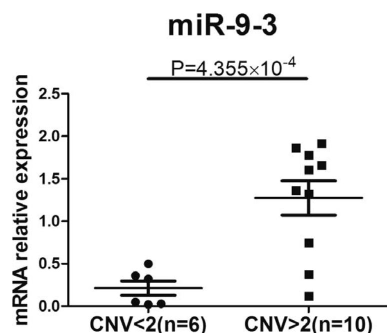

FIGURE 2. The influence of CNVs in miR-9-3 on the mRNA levels in PBMCs. The mRNA expression of miR-9-3 in PBMCs from healthy controls with different gene copies. Significance was tested by SPSS's two independent samples nonparametric test.

miR-9-3 Regulates the Expression of IL-1β and IL-6 in hRPE Cells

The cytokine production of transfected hRPE cells with either miR-9-3 mimics or inhibitors was measured at the protein level. Interleukin 6 and IL-1β production of LPS-treated hRPE was significantly increased in the 50 nM miR-9-3 mimic–transfected group as compared with the mimic controls (Fig. 4). Transfection of hRPE cells with the inhibitor (100 nM) resulted in a downregulated expression of IL-6, but had no effect on IL-1β (Fig. 4).

The Relationship Between miR-9-3 and IL-4

To elucidate the target of miR-9-3, the target gene of miR-9-3 was predicted by using a website (www.targetscan.org; provided in the public domain by Whitehead Institute, Cambridge, MA, USA) that described a high score between miR-9-3 and IL-4. The production of IL-4 was therefore also examined and showed a significant decrease in the 50 nM miR-9-3 mimic–transfected group compared with mimic controls (Fig. 5). A luciferase reporter assay was further performed to verify this conjecture. Unfortunately, hsa-miR-9-3p did not

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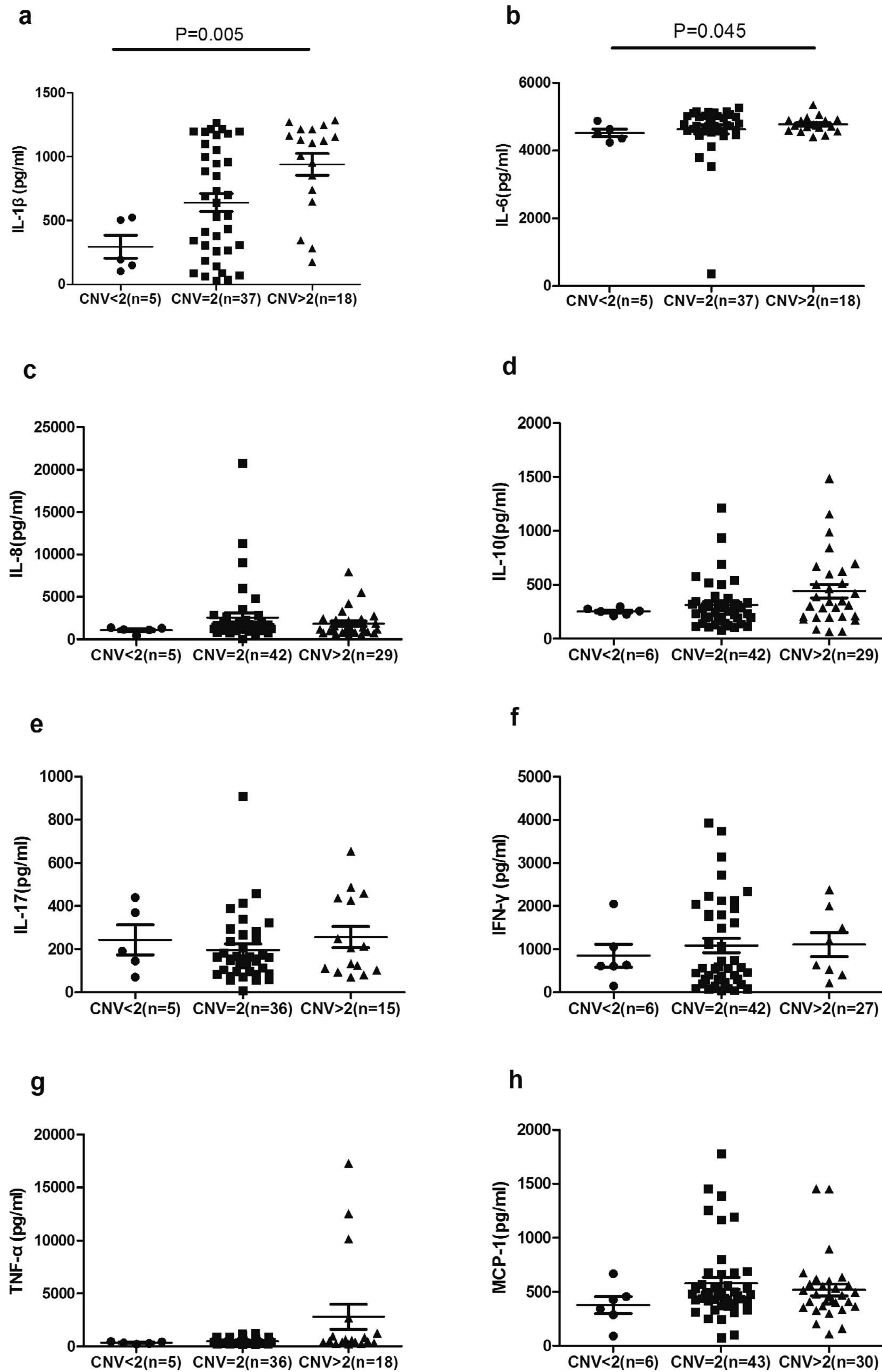

FIGURE 3. The influence of miR-9-3 CNVs on cytokine production in PBMCs. The levels of IL-1β (**a**), IL-6 (**b**), IL-8 (**c**), IL-10 (**d**), IL-17 (**e**), IFN-γ (**f**), TNF-α (**g**), and MCP-1 (**h**) in PBMCs from normal controls having different gene copy numbers of miR-9-3 (CNV $<$ 2: $n = 5$–6, CNV = 2: $n = 36$–42, CNV $>$ 2: $n = 15$–29). Significance was tested by two independent samples nonparametric test or 1-way ANOVA test.

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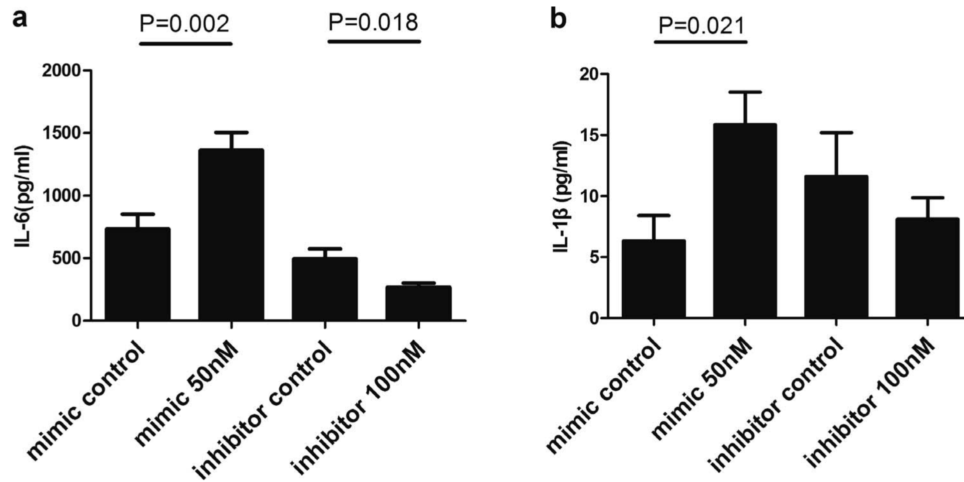

Figure 4. The influence of miR-9-3 mimics and inhibitors on cytokine production in transfected hRPE cells. The IL-6 and IL-1β production of LPS-treated hRPE was significantly increased in the miR-9-3 mimic (50 nM)–transfected group compared with mimic controls. Transfection of hRPE with inhibitor (100 nM) showed downregulated expression of IL-6 but did not affect IL-1β. Significance was tested by paired samples test.

show any significant interaction with 3′-UTR of the IL-4 gene in either ARPE-19 or hRPE cells (Fig. 6).

Discussion

To our knowledge this is the first report on the association of miRNA CNVs with AAU with or without AS. This study showed that miR-9-3, miR-301a, and miR-143 CNVs have a significant association with AAU^+AS^+, but not with AAU^+AS^-. We furthermore observed that miR-23a, miR-146a, and miR-205 CNVs were consistently associated with the susceptibility to both AAU^+AS^- and AAU^+AS^+.

Since the frequency of low copy numbers of miR-143 and miR-146a is less than 1% in controls, the number of individuals having fewer than two copies was limited to one of our healthy subjects. We therefore chose miR-9-3, which has a higher frequency of low copy numbers in controls, to examine the further functional role between its CNV and mRNA expression and also the effect of miR-9-3 CNV on the production of inflammatory cytokines. The miR9-3-level in PBMCs from controls was positively related to its copy numbers. The mRNA expression of miR-9-3 was significantly decreased in both AAU^+AS^+ and AAU^+AS^-, yet CNV was only associated with AAU^+AS^+, suggesting that these diseases may show disease heterogeneity related to the role of miR-9-3 in their pathogenesis. Since there are three independent miR-9 precursors (miR-9-1, miR-9-2, and miR-9-3) that encode the same mature miR-9 in the human genome,[41] miR-9-3 may have a similar biological function as miR-9, which might also explain the observed discrepancy. A previous study[28] has shown that a low level of miR-9 increases the expression of NF-κB, which plays an important role in the pathway of inflammation. Additionally, the expression of NF-κB is found in the retina of experimental autoimmune uveoretinitis (EAU) mice but not in control mice,[42] suggesting that NF-κB is involved in EAU. It has also been reported that NF-κB is highly expressed in the T-cell nucleus of Behcet's patients but hardly in normal human T cells.[43] Whether the association with miR-9-3 observed in our study is due to an effect mediated by regulating the expression of NF-κB is not clear and deserves further study. Further functional studies were performed to explore the role of miRNA CNVs in the pathogenesis of these diseases. The results showed a decreased production of IL-1β and IL-6, but not IL-8, IL-10, IL-17, IFN-γ, TNF-α, and MCP-1 by LPS-stimulated PBMCs in the group having fewer than two copies of miR-9-3. Since hRPE cells could possibly be involved in the pathogenesis of uveitis,[44] we also performed a number of experiments with these cells. Consistent with the results seen with PBMCs, the IL-1β and IL-6 levels of hRPE transfected with miR-9-3 mimics and inhibitors were upregulated in the mimics group and downregulated in the inhibitor group, respectively. Our data concerning IL-Iβ and IL-6 production were consistent with the positive regulation of IL-1β and IL-6 by miR-9 found by previous studies.[45,46] Although high local levels of IL-6 have been associated with both clinical as well as experimental uveitis,[47,48] suggesting a proinflammatory effect, IL-6 may also have the ability to inhibit inflammation.[49,50] Steensberg et al.[51] have found that physiological concentrations of IL-6 are anti-inflammatory rather than proinflammatory. Another study (Wang X, et al. *IOVS* 2016; 57:ARVO E-Abstract 5382) also demonstrates that IL-6 signaling has an inhibitory effect on inflammation and neuroprotection in retinal detachment. At first sight our data might thus appear to raise confusion, since the low miR-9-3 CNVs seen in our patients is associated with a lower IL-6 expression, which appears paradoxical. As men-

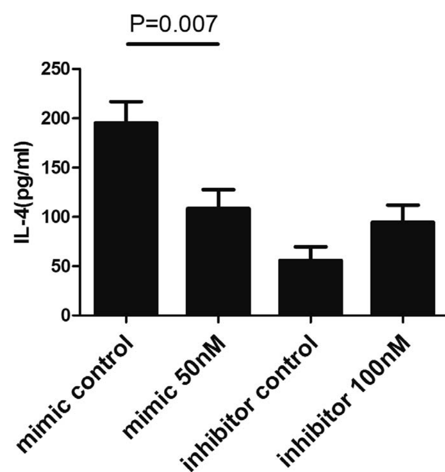

Figure 5. The influence of miR-9-3 mimics and inhibitors in hRPE cells on cytokine production of IL-4. The IL-4 production was significantly decreased in the miR-9-3 mimic (50 nM)–transfected group compared with mimic controls. Significance was tested by paired samples test.

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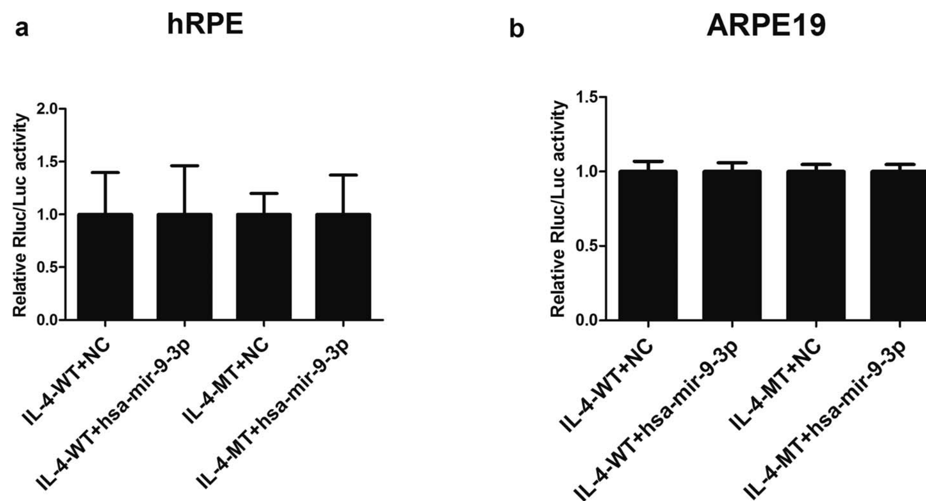

FIGURE 6. Dual luciferase reporter assay in hRPE and ARPE-19 cells. (**a**) Dual luciferase assay of hRPE cells cotransfected with the firefly luciferase constructs containing the IL-4-wild type (IL-4-WT) or IL-4-mutant (IL-4-Mut) 3′-UTR and miR-9-3 mimics or its negative control (NC). (**b**) Dual luciferase assay of hRPE cells cotransfected with the firefly luciferase constructs containing the IL-4-WT or IL-4-Mut 3′-UTR and miR-9-3 mimics or its NC. Significance was tested by paired samples test.

tioned above there are now numerous studies suggesting that IL-6 may also have an anti-inflammatory function. The lack of IL-6 seen in our patients with low miR-9-3 CNVs could thus be explained by a dysregulated control of inflammation by IL-6. Furthermore, we would like to favor the theory that low expression of miR-9 leads to disease rather than disease leading to low expression.

Animal models have shown that miRNAs play important regulatory roles by targeting mRNAs for cleavage or translational repression.[16] The target site of miR-9-3 has been investigated and shown to have a binding site with several genes including 3′-UTR of IL-4 (www.targetscan.org), and further evidence shows that IL-4 has the ability to suppress the production of IL-1β and IL-6.[52–54] Therefore, we firstly detected the relationship between IL-4 and miR-9-3 by a dual luciferase reporter study. Unfortunately, no significant interaction was found between 3′-UTR of the IL-4 gene and hsa-miR-9-3p, suggesting that there were other potential pathways for inhibition of IL-1β and IL-6 expression. Further studies need to be performed to explore the exact role of the miR-9-3 pathway in uveitis.

We did not focus on the role of HLA-B27, since a larger group of patients would be needed. Furthermore, not all patients had been tested for HLA-B27 for logistic or economic reasons and because it is not yet included in the current diagnostic criteria. For a proper analysis, the HLA-B27 status of the controls would also be needed. Despite these drawbacks, we did perform a preliminary study to investigate the effect of HLA-B27 on miRNA CNV frequencies in our patient groups. Associations found in the whole group of patients could be confirmed in the HLA-B27$^+$ patients, but not always in the HLA-B27$^-$ negative group. This is probably caused by the low number of patients in the latter group.

A possible limitation of our study might be the fact that some of our AAU patients might develop AS at a later time after entering the study. The age of onset of AAU$^+$AS$^+$ patients reported in literature ranges between 15 and 40 years.[55] The mean age of the AAU$^+$AS$^-$ patients enrolled in our study was 39.6 ± 12.9 years, suggesting that these patients will have a low probability of developing AS later in life, although we cannot definitely exclude it. Further longitudinal studies or a study in an even older patient group may solve this limitation.

In conclusion, we found that low copy numbers of miR-9-3 and miR-143, and high copy numbers of miR-301a, confer risk for AAU$^+$AS$^+$ but not for AAU$^+$AS$^-$. Additionally, the increased frequency of more than two copies for miR-23a and fewer than two copies for miR-146a was consistently linked to the susceptibility to both AAU$^+$AS$^-$ and AAU$^+$AS$^+$. The significantly decreased mRNA expression of miR-9-3 in AAU$^+$AS$^+$ was consistent with the increased frequency of low copy numbers in the patient groups. The mechanism of miR-9-3 in AAU$^+$AS$^+$ pathogenesis may involve a response to triggers leading to a lower IL-6 expression.

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