HHS Public Access

Author manuscript

Environ Mol Mutagen. Author manuscript; available in PMC 2017 January 01.

Published in final edited form as:

Environ Mol Mutagen. 2016 January ; 57(1): 58–64. doi:10.1002/em.21982.

Functional Single Nucleotide Polymorphism in *IL-17A* 3′ Untranslated Region is Targeted by miR-4480 *in vitro* and may be Associated with Age-Related Macular Degeneration

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Abstract

Age-related macular degeneration (AMD) is a leading cause of irreversible central vision loss in the elderly. Genetic factors contributing to AMD include single nucleotide polymorphisms (SNPs) in immune-related genes including *CFH, C2, CFI, C9*, and *C3*, thus implicating these pathways in AMD pathogenesis. MicroRNAs (miRNAs) are powerful regulators of gene expression and execute this function by binding to the 3′ untranslated region (3′UTR) of target mRNAs, leading to mRNA degradation. In this study, we searched for the possible association of SNPs in the 3′UTR region of *IL-17A*, a gene implicated in AMD pathogenesis without any previous SNP association with AMD. Using two independent sample cohorts of Caucasian subjects, 6 SNPs in the IL-17A 3′-UTR were selected for genotyping based on bioinformatic predictions of the SNP effect on microRNA binding. The SNP rs7747909 was found to be associated with AMD ($p < 0.05$) in the NEI cohort, using a dominant model logistic regression. Luciferase reporter gene assays and RNA electrophoretic mobility shift assays were performed using ARPE-19 cells to confirm the preferential binding of microRNAs to the major allele of the SNP. Our findings support the hypothesis that microRNA-mediated gene dysregulation may play a role in the pathogenesis of AMD.

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Statement of Author Contributions

Jingsheng Tuo conceived of and proposed the study. Nicholas A. Popp, Dianke Yu, Baiting Ning, Chi-Chao Chan, and Jingsheng Tuo designed experiments. Emily Y. Chew, Chi-Chao Chan, and Jingsheng Tuo gathered human participants for the gene association studies and received IRB approval. Nicholas A. Popp, Dianke Yu, and Bridgett Green performed experiments. All authors analyzed the results. Nicholas A. Popp and Dianke Yu wrote the manuscript. All authors provided insight on, revised, and approved the manuscript.

Keywords

microRNA; age-related macular degeneration; inflammation; IL-17A; SNPs; epigenetics; genetics

Introduction

Age-related macular degeneration (AMD) is a leading cause of central blindness in people over the age of 50, with the most recent estimates of its prevalence globally to be 8.69% with approximately 196 million people expected to be affected by 2020 [Wong, et al. 2014]. People of European ancestry are the highest at risk for developing AMD, with prevalence estimates for people over the age of 50 as high as 12.33% for any AMD [Wong, et al. 2014]. Despite the immense amount of work on the pathogenesis of AMD, much remains to be understood about the genetic and environmental components of its etiology. Currently identified single nucleotide polymorphisms (SNPs) associated with AMD can only explain 28–43% of the variance of AMD disease in patients, and many of the mechanisms through which these SNPs function to lead to AMD remain unknown [Fritsche, et al. 2014].

Most SNPs associated with AMD have been found in the coding sequences of genes, with the remaining SNPs found in promoters and introns. However, very few SNPs in the 3′ untranslated region (3′UTR) have been associated with AMD, despite these SNPs' potential to effectively disrupt microRNA (miRNA) binding and facilitate mRNA degradation, a mechanism found to be in play in other diseases [Abelson, et al. 2005; Chang and Mendell 2007]. Further, RNA processing defects via DICER and accumulation of *Alu* RNAs have been connected to the geographic atrophy (GA) form of late stage AMD [Kaneko, et al. 2011; Tarallo, et al. 2012].

To pursue this mechanistic idea, we hypothesized that genes implicated in AMD could contain particular variations in their 3′UTR, thus altering post-transcriptional gene regulation and contributing to AMD pathogenesis. We chose *IL-17A* due to the following reports of its involvement in AMD. In addition to the known role of IL-17A in autoimmune uveitis in the eye [Amadi-Obi, et al. 2007; Luger, et al. 2008], patients with AMD show increased serum concentrations of IL-17A, which can be induced *in vitro* by complement C5a, as well as aberrant *IL-17A* mRNA and protein expression in AMD macular lesions [Liu, et al. 2011; Ardeljan, et al. 2014]. Further, the mRNA and protein of IL-17RC, the receptor for IL-17A, is elevated in these same macular lesions [Wei, et al. 2012]. Injection of soluble IL-17A receptor into the eyes of $Ccl2^{-/-}/Cx3cr1^{-/-}$ mice, a murine model of AMD slows the progression of their AMD-like pathologies [Ardeljan, et al. 2014]. As such, the role of IL-17A-induced pathology in AMD is well-established, yet a causative mechanism remains elusive. This could be explained by allele specific dysregulation of miRNA binding ability in the 3′UTR of IL-17A in AMD, a hypothesis we sought to test.

Methods

Study Subjects

All research followed the tenets of the Declaration of Helsinki and the study protocols were approved by the Institutional Review Boards of the participating institutions (National Eye Institute (NEI) and 11 retina specialty clinical centers for the Age-Related Eye Disease Study (AREDS)). Demographics of the two study groups are summarized in Table 1. Only Caucasian patients with late stage AMD (defined as geographic atrophy or neovascular AMD) were included in the study. Methods for the selection and evaluation of patients have been previously defined and are briefly described here [Age-Related Eye Disease Study Research Group 2000; Clemons, et al. 2005; Davis, et al. 2005; Ardeljan, et al. 2013].

NEI ophthalmologists evaluated patients and control subjects using the AREDS criteria for both the NEI and AREDS subjects. Clinical ophthalmoscopy and fundus grading were performed on all subjects; controls presented with fewer than 5 small drusen (< 63 mm in diameter) and no other signs of retinal disease [Age-Related Eye Disease Study Research Group 2000; Age-Related Eye Disease Study Research Group 2001]. In this study, only DNA from participants self-identifying as non-Hispanic Caucasian were genotyped.

SNP Selection and Genotyping

We used the miRNASNP 2.0 database to predict the effect of SNPs in *IL-17A* on miRNA binding strength [Gong, et al. 2012]. Six SNPs were selected accordingly. The selected SNPs in *IL-17A* were genotyped using Taqman SNP Genotyping Assays (Applied Biosystems, Foster City, CA).

miRNA selection

miRNASNP 2.0 predicted that rs7747909 is located in the binding sites of miR-548s and miR-4480 in IL-17A mRNA. We used the RNAhybrid program to calculate the free energy of binding between hsa-miR-548s or hsa-miR-4480 and the *IL-17A* mRNA harboring rs7747909 G or A allele sequences, respectively [Rehmsmeier, et al. 2004].

Luciferase Reporter Gene Assay

Cloning primers IL-17A-F: 5′-GGG AAA GUC ACA CTC CCC AAA GCA GTT AG -3′ and IL-17A-R: 5′-GGA GAC AUA ATC TTT CTT TTC AGC CAT GTT -3′, were used to amplify the core region of *IL17A* 3′UTR harboring the rs7747909 G allele sequence. PCR products were then digested with USER enzyme (New England Biolabs, Ipswich, MA), and subcloned into the linearized nicked pGL3-CU vector [Yu, et al. 2015]. The resultant plasmid was designated as IL17A-G-CU. IL17A-A-CU construct, containing the rs7747909 A allele sequence, was generated by site-directed mutagenesis using IL17A-A-F: 5′-AGA GGT AAC ACT TAG CCA AGA TAT GAG AT -3′ and IL17A-A-R: 5′-ATC TCA TAT CTT GGC TAA GTG TTA CCT CT -3′ primers. Both constructs were restriction-mapped and sequenced to confirm the authenticity.

ARPE-19 (ATCC, Manassas, VA), a human retinal pigmented epithelium cell line, was cultured in Dulbecco's Modified Eagle medium with 10% FBS, and seeded at 4×10^4 cells

per well in 96-multiwell plates. Once grown to 70%–80% confluence, cells were transfected with the constructed IL17A-G-CU or IL17A-A-CU (100 ng), together with 50 nmol/L hsa-miR-548s mimic, hsa-miR-4480 mimic, or miRNA negative control (Thermo Scientific, Tewksbury, MA), respectively, using Lipofectamine 2000 reagent (Life Technologies, Carlsbad, CA). Transfection experiments were carried out at least 3 times, and each was performed in a triplicate manner.

RNA Electrophoretic Mobility Shift Assay (RNA EMSA)

All oligonucleotides were synthesized by Integrated DNA Technologies (Coraville, IA). The 2′ O-Methyl modified mRNA oligonucleotides IL17A-G: 5′-CUU CAG AGG UAA CAC UUG GCC AA -3′, and IL17A-A: 5′-CUU CAG AGG UAA CAC UUA GCC AA -3′, which are mRNA fragments containing the G allele or A allele of rs7747909, respectively, were labeled by IRDye®800 dye in their 5′ ends. The miRNA oligonucleotides hsa-miR-548s: 5′-AUG GCC AAA ACU GCA GUU AUU UU -3′ and hsa-miR-4480: 5′-AGC CAA GUG GAA GUU ACU UUA -3′ were 5′ labeled by cy5.5™. Cytoplasmic extracts were prepared from ARPE-19 cells according to the NE-PER Nuclear and Cytoplasmic Extraction Reagents protocol (Thermo Scientific).

RNA Electrophoretic Mobility Shift Assays were performed as described previously [Yu, et al. 2015]. Briefly, synthetic miRNA and mRNA oligonucleotides were heated for 5 minutes at 80°C and placed on ice to relax RNA folding, and then incubated at 25°C for 20 min. Cytoplasmic extracts (2 μg), and tRNA (1 μg) as nonspecific competitor RNA, were added into the reaction mixture to test the RNA:protein interactions. Antibodies against Ago1 or Ago2 were purchased from Abcam (Cambridge, MA), and applied in the supershift assays. The reaction mixture was separated on a 12% PAGE by electrophoresis, and the resultant mobility shifts were detected by Odyssey CLx Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).

Statistical Analysis

SNP association analyses were performed using SVS software (version 7.4.1, HelixTree Genetics Analysis Software, Golden Helix Inc., Bozeman, MT, USA). A dominant model (two major alleles vs. at least one minor allele [dd or Dd] vs. DD) was analyzed using logistic regression adjusted for age, gender, and smoking status. χ^2 tests were used to determine the *p*-values and odds ratios (OR) of the associations.

Results

The SNP rs7747909 in the *IL-17A* Gene is Associated with Increased Risk for Late-Stage AMD

The results from the SNP association tests and logistic regression in the NEI sample set are given in Table 2. Initial analysis confirmed two SNPs in *IL-17A* were significantly associated with an increased risk of developing AMD. rs7747909 showed a strong association ($p = 0.0022$; OR = 1.73; 95% CI: 1.22 – 2.45) whereas rs10484879 was less strong but still below the $p < 0.05$ threshold ($p = 0.0257$; OR = 1.48; 95% CI: 1.05 – 2.08).

As can be seen in Figure 1, rs10484879 was found to be in linkage disequilibrium with rs7747909 (D′ = 0.999045).

After using logistic regression to correct for the potential confounders of age, smoking status, and gender, only rs7747909 remained significantly associated with AMD and suggested a pathogenic role in the development of AMD (p = 0.0308; OR = 1.72; 95% CI: 1.05 – 2.83). rs10484879 in *IL-17A* did not maintain statistical significance after logistic regression ($p = 0.0878$). Interestingly, *IL-17A* SNP rs3748067, which was recently reported to be associated with AMD in a Chinese cohort, [Zhang, et al. 2014] did not show an association in our cohort ($p = 0.5499$).

Based on the findings in the NEI cohort, rs7747909 was then genotyped in the AREDS cohort for replication purposes. However, dominant logistic analysis failed to find any association between rs7747909 and AMD in AREDS sample set (Table 2; p > 0.05).

Allele Specific Suppression of *IL-17A* by hsa-miR-548s and hsa-miR-4480

RNAhybrid program predicted that the G-allele-harboring sequence exhibited a lower free energy than the A-allele-harboring sequence to bind either hsa-miR-548s (−20.1 kcal/mol vs −14.0 kcal/mol) or hsa-miR-4480 (−23.0 kcal/mol vs −16.5 kcal/mol). This prediction suggests rs7747909 G allele mRNA sequence is more likely to be targeted by hsa-miR-548s or hsa-miR-4480. The reporter gene constructs retaining the core region of IL-17A 3′UTR and harboring the rs7747909 G or A allele sequence, were transfected into ARPE-19 cells, together with the microRNA hsa-miR-548s or hsa-miR-4480 mimics, or negative control, respectively. Compared with the microRNA negative control, hsa-miR-548s or hsa-miR-4480 efficiently suppressed the expression of G-allele-harboring luciferase (41% or 40%, all $p < 0.001$), respectively, but no significant suppression effect was observed in the expression of A-allele-harboring luciferase (Figure 2C).

Allele Specific Interaction between IL-17A mRNA and hsa-miR-548s or hsa-miR-4480

As shown in Figure 3A, a complex formed by the interaction between hsa-miR-4480 and its cognate mRNA oligonucleotides of IL-17A 3′UTR harboring the rs7747909 G allele sequence (lane 8) was observed *in vitro*, but no such an interaction was observed between hsa-miR-4480 and the mRNA oligonucleotides of IL-17A 3′UTR harboring the rs7747909 A allele sequence (lane 7). In addition, ARPE-19 cytoplasmic extract was able to bind the complex formed by G-allele-harboring oligonucleotides and hsa-miR-4480 to form a RNA-protein complex (Figure 3B, lane 2). Further, Ago1 antibody could bind to above RNA-protein complex to reform a new supershift complex (RNA-protein-antibody) (Figure 3B, lane 4), indicating that Ago1 might contribute to the suppression effect of hsa-miR-4480 on the production of IL-17A, which was G-allele dependent. However, neither RNA-protein complex (Figure 3C, lane 1 and 2), nor RNA-protein-antibody complex (Figure 3C, lane 3,4,5 and 6) between hsa-miR-548s and rs7747909-harboring mRNA (Figure 3A, lane 5 and 6) was observed in the RNA EMSAs, no matter which allele of rs7747909 in IL-17A 3′UTR mRNA was applied in the assays, probably due to the fact that hsa-miR-548s was easily dimerized under our experimental conditions (Figure 3A, lane 3).

Discussion

Genetic changes in miRNAs and miRNA binding sites of gene transcripts have potential to affect disease susceptibility, yet despite convincing evidence for miRNA involvement in inflammation, angiogenesis, and ocular disease, their role in AMD has not been explored to a great extent [Shen, et al. 2008; Sonkoly, et al. 2008; Bazzoni, et al. 2009; Lin, et al. 2011; Sabatel, et al. 2011; Wang, et al. 2012; Tuo, et al. 2014]. While single SNPs in miRNAs have the potential to affect the post-transcriptional RNA processing of tens to hundreds of genes, they are relatively depleted across the genome [Hu and Bruno 2011] SNPs in the 3′UTR miRNA binding regions, instead, have the opportunity to modulate miRNA-mediated gene expression [Chang and Mendell 2007].

Evidence of the pathogenic role of IL-17A in AMD without a known genetic component led us to search for SNPs in *IL-17A* that influence the development of AMD in our Caucasian NEI cohort [Tuo, et al. 2006; Tuo, et al. 2008; Ardeljan, et al. 2013; Chu, et al. 2014]. Out of 6 SNPs tested, we found evidence of a single SNP, rs7747909, in the 3′UTR of *IL-17A* that associates with AMD with a dominant logistic model. However, in the second replication cohort of Caucasian AREDS patients, which has a similar number subjects as the NEI cohort, the SNP association did not replicate with significance. This lack of concordance between the NEI and AREDS cohorts has been noted previously [Ardeljan, et al. 2013]. Lack of replication between studies, however, is common, even with highly associated SNPs, particularly in complex diseases where multiple genetic factors are likely at play [Hirschhorn, et al. 2002; Gorroochurn, et al. 2007; Greene, et al. 2009]. In one meta-analysis of 166 genetic associations across a variety of diseases, only 6 associations replicated consistently over a minimum of three cohorts, though 97 replicated at least once [Hirschhorn, et al. 2002]. The difficulty in replication likely comes from heterogeneity between cohorts and weak genetic effects that can be easily masked by unknown cofounders [Hirschhorn, et al. 2002; Gorroochurn, et al. 2007]. Recent work, though, suggests that failure to replicate may indicate gene epistasis; small changes in SNPs from other genes between cohorts can lead to a failure to replicate and can suggest gene-gene interactions [Greene, et al. 2009].

To check whether there could be functional consequences to the rs7747909 SNP in AMD, we showed using luciferase assays that rs7747909 in the 3′UTR of *IL-17A* can modify the binding ability of hsa-miR-4480 and hsa-miR-548s in an allele-specific manner and alter degradation of *IL-17A* mRNA *in vitro*. RNA EMSA showed complex formation of hsa-miR-4480 and the major G allele of rs7747909 but not with the minor A allele. This complex could also bind Ago1 but not Ago2, indicating a distinct functional mechanism for cleaving *IL-17A* mRNA in the cytoplasm [Ender and Meister 2010; Turchinovich and Burwinkel 2012]. Intriguingly, hsa-miR-548s did not directly form a complex with either allele of IL-17A (Figure 3A). In addition, hsa-miR-548s together with IL-17A could not recruit Argonaut proteins to form a RNA-induced silence complex under our experimental conditions (Figure 3C). A reasonable explanation is that the self-dimerization of hsa-miR-548s (Figure 3A, Lane 3) probes disturbs the interaction between hsa-miR-548s and target mRNA or protein complex. However, the data cannot exclude another possibility that

the hsa-miR-548s molecular truly fails to bind IL-17A mRNA directly or indirectly (mediated by ribonucleoproteins).

In conclusion, our data suggest that rs7747909 in *IL-17A* may be associated with development of AMD in a Caucasian cohort and that this SNP can alter the ability of hsa-miR-4480 to bind and degrade *IL-17A* mRNA *in vitro*. However, since miRNAs can have many mRNA targets, the bioinformatic prediction of hsa-miR-4480 to IL17A 3′UTR does not exclude its possible binding to other genes in the IL17A pathway, thus regulating IL-17A expression indirectly. Nevertheless, because of the robust post-transcriptional regulation of *IL-17A* by rs7747909 and the current understanding that IL-17A is an important effector of multiple pathways implicated in AMD pathogenesis such as the NLRP3 inflammasome and complement, [Lalor, et al. 2011; Liu, et al. 2011; Tarallo, et al. 2012; Marneros 2013] changes to miRNA-mediated mRNA degradation may alter IL-17A response in AMD; as such, the genetic and functional contribution of IL-17A to AMD pathogenesis warrants further investigation.

Acknowledgments

The NEI Intramural Research Program provided funding support. The authors would like to thank the participants and their families for enrolling in this study.

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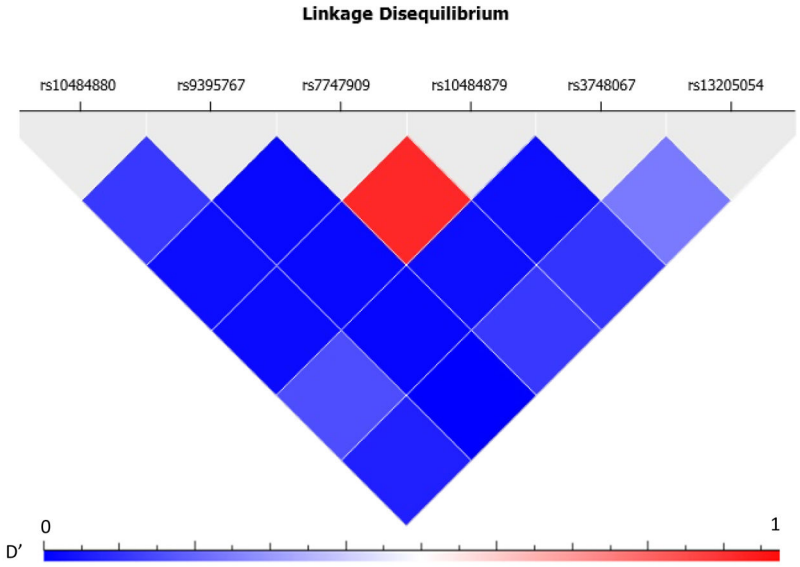

Figure 1.
Linkage disequilibrium map of the *IL-17A* 3′UTR showing the degree of linkage disequilibrium between the 6 genotyped SNPs.

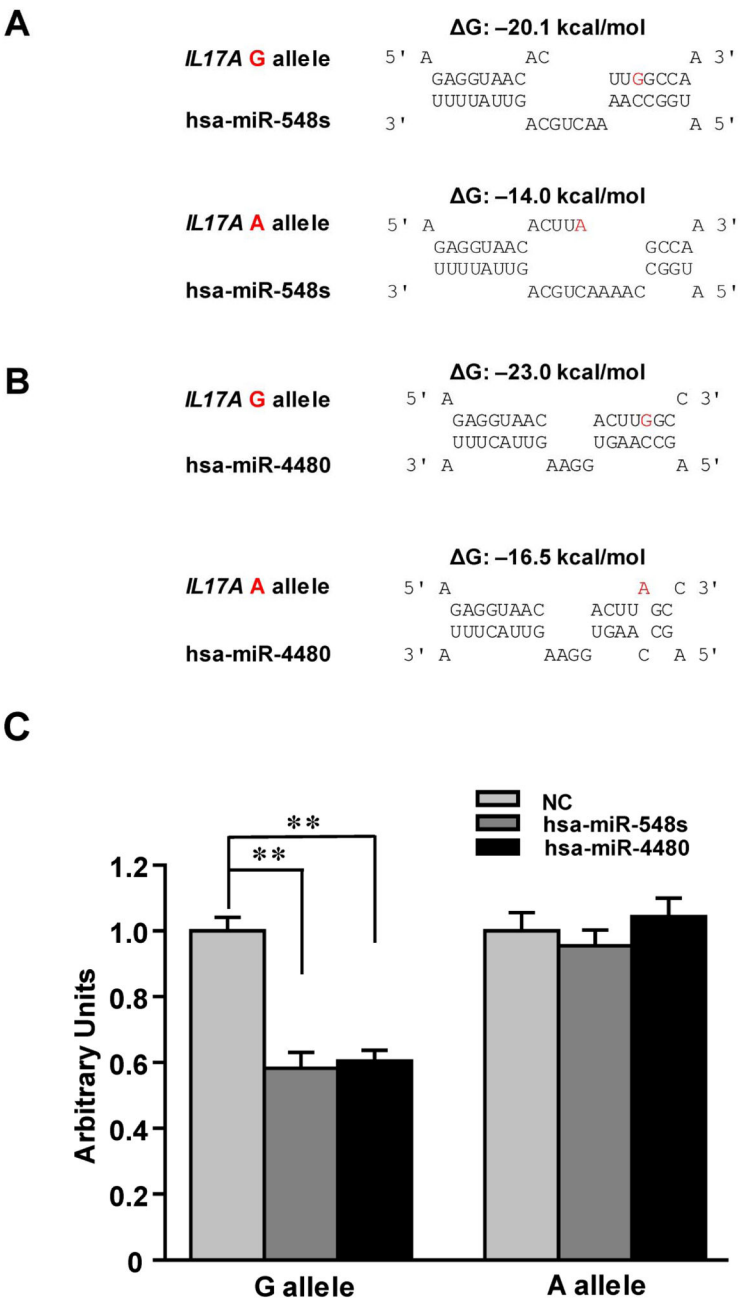

Figure 2.

hsa-miR-548s and hsa-miR-4480 suppress *IL-17A* gene expression in an allele specific manner. (A) Targeting prediction of hsa-miR-548s to the 3′UTR of *IL-17A* based on rs7747909 allele. The binding energy of the miRNA to the major G allele is stronger than the minor A allele. (B) Targeting prediction of hsa-miR-4480 to the 3′UTR of *IL-17A* based on rs7747909 allele. The binding energy of the miRNA to the major G allele is stronger than the minor A allele. (C) Luciferase reporter assay to investigate the effect of each allele of rs7747909 in ARPE-19 retinal pigmented epithelium cells. ARPE-19 cells were transiently transfected with a plasmid containing either the G or A allele for rs7747909 together with

either hsa-miR-548s, hsa-miR-4480, or a miRNA negative control (NC). *Renilla* luciferase was measured and compared to firefly luciferase in three independent experiments. * $p < 0.05$.

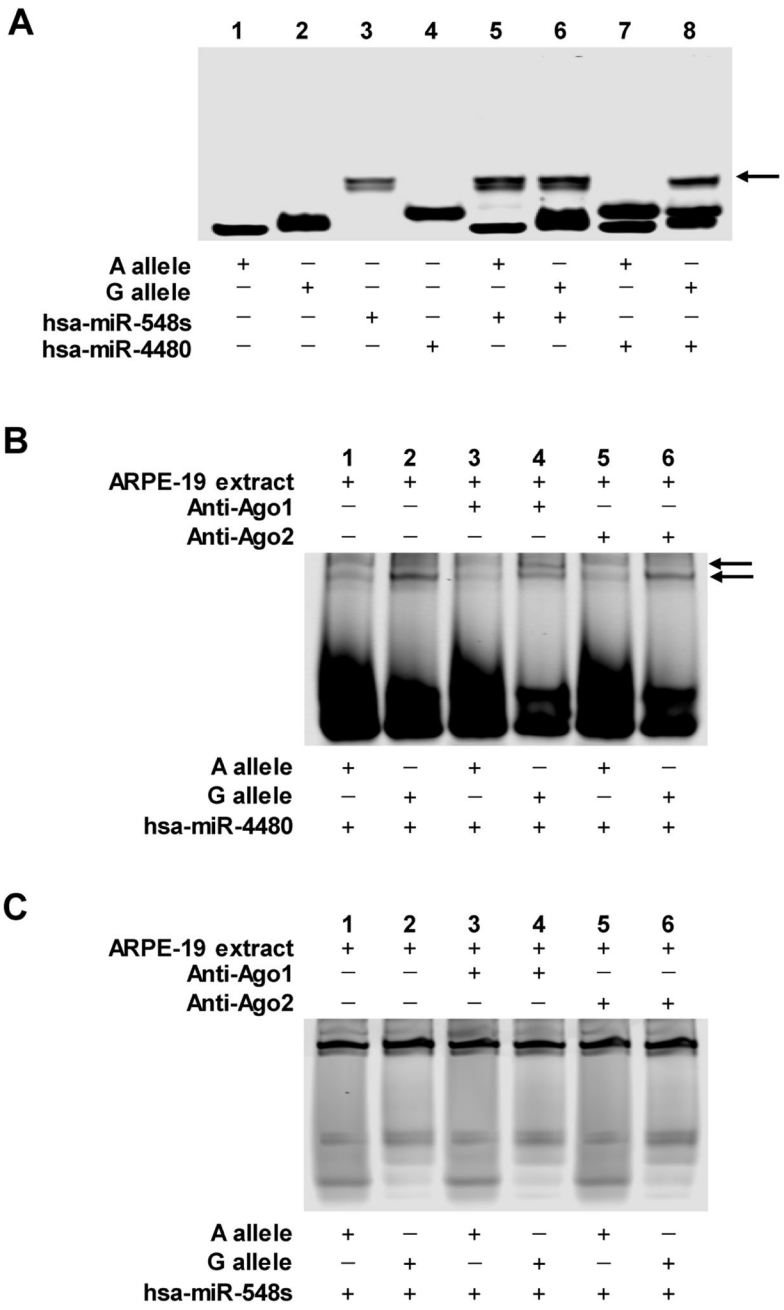

Figure 3.

Interaction of hsa-miR-548s and hsa-miR-4480 with *IL-17A* counterparts. (A) RNA EMSA was used to detect the formation of miRNA-mRNA complexes *in vitro*. Lanes 1–4 show the mobility of the labeled nucleotides. Lanes 5–6 show the mobility of hsa-miR-548s with each allele of rs7747909 in *IL-17A*. Lanes 7–8 show the mobility of hsa-miR-4480 with each allele of rs7747909 in *IL-17A*. (B) Addition of ARPE-19 cytoplasmic extracts to hsa-miR-4480 and *IL-17A* alleles shows formation of a RNA-protein complex (lanes 1–2). Ago1 (lanes 3–4) and Ago2 (lanes 5–6) antibodies were used to probe for Ago1 or Ago2 involvement in this RNA-protein complex via the formation of a supershift complex. (C)

Addition of ARPE-19 cytoplasmic extracts to hsa-miR-548s and *IL-17A* alleles shows no formation of a RNA-protein complex (lanes 1–2). Ago1 (lanes 3–4) and Ago2 (lanes 5–6) antibodies were used to probe for Ago1 or Ago2 involvement in this RNA-protein complex via the formation of a supershift complex.

Table 1

Subject demographics in the NEI and AREDS cohorts

	NEI		AREDS	
	Case	Control	Case	Control
	(N = 247)	(N = 162)	(N = 297)	(N = 193)
Mean Age (SD)	78.8 (8.3)	66.4 (10.7)	79.2 (5.0)	76.8 (4.3)
Female N (%)	133 (53.8)	94 (58.0)	176 (59.3)	105 (54.4)
Male N (%)	114 (46.2)	68 (42.0)	121 (40.7)	88 (45.6)
Never smoking N (%)	118 (47.8)	85 (52.5)	118 (39.7)	94 (48.7)
Former or Current smoking N (%)	129 (52.2)	77 (47.5)	179 (60.3)	99 (51.3)

Abbreviations: AREDS: Age-Related Eye Disease Study, NEI: National Eye Institute.

Table 2

Association of 6 *IL-17A* polymorphisms with AMD in NEI and AREDS cohorts

	MAF (%)		Uncorrected Model					Dominant Model		
	Control	Case	*P*	OR	95% CI	HWE *P*	FDR	*P*/PA	ORA	95% CI
NEI										
rs3748067	9.8	7.0	0.1587	0.70	(0.42–1.16)	0.6679	0.3471	0.5499	0.82	(0.43–1.57)
rs7747909	17.6	27.0	0.0022	1.73	(1.22–2.45)	0.8347	0.0126	0.0308	1.72	(1.05–2.83)
rs9395767	38.6	39.7	0.7613	1.05	(0.78–1.40)	0.2451	0.8595	0.7782	1.07	(0.66–1.75)
rs10484879	19.3	26.1	0.0257	1.48	(1.05–2.08)	0.9374	0.0817	0.0878	1.53	(0.94–2.51)
rs10484880	7.5	7.1	0.8044	0.93	(0.55–1.60)	0.9158	0.8798	0.4226	0.76	(0.39–1.49)
rs13205054	30.7	24.9	0.0704	0.75	(0.55–1.03)	0.8258	0.1760	0.3215	0.79	(0.49–1.27)
AREDS										
rs7747909	25.4	25.4	0.9910	1.00	(0.75–1.34)	0.8800	0.9910	0.6960	0.93	(0.63–1.35)

rs7747909 and rs10484879 in *IL-17A* were found to be associated with AMD in the NEI cohort ($p < 0.05$). After logistic regression on the dominant model ({dd or Dd} vs. DD), correcting for age, smoking status, and gender, only rs7747909 in *IL-17A* had a *p*-value less than 0.05. rs7747909 did not replicate in the AREDS cohort.

Abbreviations: AREDS: Age-Related Eye Disease Study, CI: Confidence interval, FDR: False discovery rate, HWE: Hardy-Weinberg equilibrium, MAF: Minor allele frequency, NEI, National Eye Institute, OR: Odds ratio.