A Genetic Variant in the *SKIV2L* Gene Is Significantly Associated With Age-Related Macular Degeneration in a Han Chinese Population

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Purpose. Previous studies have shown that genetic variants in the complement component 2 (*C2*)/complement factor B (*BF*) gene are associated with AMD in Caucasians, but not in Han Chinese. Recent studies have indicated that genetic variants in the neighboring superkiller viralicidic activity 2-like (*SKIV2L*) gene showed significant association with AMD. We conducted this study to investigate whether genetic variants in the *SKIV2L* gene are associated with AMD in a Han Chinese population.

Methods. Thirteen single nucleotide polymorphisms (SNPs) in the *C2-BF-RDBP-SKIV2L-STK19* region were genotyped by the SNaPshot method in a cohort composed of 449 patients with choriodal neovascularization (CNV) AMD and 1025 healthy controls of Han Chinese descent.

Results. Among the SNPs genotyped, *P* values of seven SNPs were less than 0.05; however, only rs429608 was found to be significantly associated with AMD after correction for multiple testing. The minor allele (A) frequency of rs429608 was 0.050 in cases and 0.089 in controls, and the *P* value was 3.76×10^{-4} (0.00489 after Bonferroni correction), with an odds ratio of 0.55 (95% confidence interval, 0.40–0.77). The *SKIV2L* gene was expressed in the human RPE, retina, and D407 (human RPE) cells, and in mouse retinas and RPE.

Conclusions. We demonstrated that the rs429608 genetic variant in the *SKIV2L* gene was significantly associated with AMD in a Han Chinese population. *SKIV2L* may play an important role in the development of AMD.

Keywords: age-related macular degeneration, SKIV2L, single nucleotide polymorphism

Age-related macular degeneration (AMD) is the leading cause of blindness in aging populations. Recent studies have indicated that both genetics and environmental factors, such as smoking, play a critical role in the development of AMD.[1–6] Oxidative damage–induced inflammation also plays an important role in the pathogenesis of this disorder.[7–9] Two genes, complement factor H (*CFH*) and age-related maculopathy susceptibility 2 (*ARMS2*)/HtrA serine peptidase 1 (*HTRA1*), have been identified as major AMD loci in different populations.[10–23] Two other genes, complement component 2 (*C2*)/complement factor B (*BF*) and complement component 3 (*C3*), were shown to be significantly associated with AMD in Caucasians and Indians.[24–28] However, the results of the association studies between genetic variants in the *C2/BF* and *C3* genes and AMD in East Asian populations, including Korean, Chinese, Japanese, and so on, were inconsistent.[29–32] Yanagisawa et al.[32] recently identified that a different SNP in the *C3* gene, rs2241394, showed significant association with wet AMD in Japanese populations.

In addition, recent studies have indicated that genetic variants in the *C2/BF* neighboring gene, superkiller viralicidic activity 2-like (*SKIV2L*), show significant association with AMD in Caucasians,[33,34] suggesting that the *SKIV2L* gene is a strong candidate for AMD at this locus. In this study, we investigated if genetic variants of the *SKIV2L* gene are significantly associated with AMD in a Han Chinese population.

Materials and Methods

Study Population

The institutional review board of the Sichuan Provincial People's Hospital approved this study. All participants gave informed consent before participation in the study. Patients with AMD and age-matched controls were recruited from the ophthalmology clinic at Sichuan Provincial People's Hospital. All participants underwent a standard examination protocol, as previously described.[18,23,35]

Table 1. Characteristics of AMD Cases and Controls in This Study

Group	No.	Male, *n* (%)	Female, *n* (%)	Age, y ± SD	Age Range, y
AMD	449	233 (51.9)	216 (48.1)	67.6 ± 10.2	45–89
Control	1025	515 (50.2)	510 (49.8)	70.8 ± 9.3	60–92

All patients were given complete ophthalmic examinations, including best-corrected visual acuity measurement, ocular tonometry, slit-lamp biomicroscopy, color fundus photographs, fluorescein angiography, optical coherence tomography (OCT), and indocyanine green angiography (ICGA). Patients with clinical features of AMD and choroidal neovascularization (CNV) (CNV from other causes was excluded) in at least one eye, with or without drusen, were diagnosed as CNV AMD.[36] Polypoidal choroidal vasculopathy (PCV) was diagnosed using ICGA, which showed a choroidal origin of polypoidal lesions. Subjects with PCV lesions in any eye were also excluded. All controls were given complete ophthalmic examinations and were included according to the following criteria: (1) 60 years or older; (2) no signs of early AMD, such as soft drusen or irregular pigmentation of the RPE in the macular area; and (3) no other major eye diseases, except for mild senile cataracts and mild refractive errors. In total, 449 CNV AMD patients and 1025 normal matched controls were recruited. Demographic information about the cases and controls are listed in Table 1.

Selection of SNPs

We selected a total of 13 single-nucleotide polymorphisms (SNPs) for genotyping (Table 2). Six of these 13 SNPS, including rs9332739, rs547154, rs4151667, rs641153, rs760070, and rs429608, were reported in previous studies to be associated with AMD.[24,33,34] The other seven haplotype-tagging SNPs were obtained from the *C2-BF-RD* RNA-binding protein (*RDBP*)-*SKIV2L*-serine/threonine kinase 19 (*STK19*) region spanning 46 kb (Chr6: 32,001,000–32,047,000) in the Han Chinese population in Beijing, China (CHB), drawn from the HapMap database (HapMap Public Release #27 on Genome Browser, http://hapmap.ncbi.nlm.nih.gov/). Several of these SNPs had been genotyped in some of the samples in our previous study.[29] Among these 13 SNPs, 3 (rs2734335, rs9332739, and rs547154) were located in the *C2* gene, 2 (rs4151667 and rs641153) were located in the *BF* gene, 3 (rs760070, rs3880457, and rs9501161) were located in the *RDBP* gene, 4 (rs437179, rs429609, rs410851, and rs2075702) were located in the *SKIV2L* gene, and 1 (rs474534) was located in the *STK19* gene. These 13 SNPs cover five genes in order.

Genotyping

Venous blood was drawn from each subject and collected in an EDTA-containing tube. Genomic DNA was extracted from the blood using a Gentra Puregene Blood DNA kit (Gentra, Minneapolis, MN). SNP genotyping was performed by the dye terminator-based SNaPshot method, as previously described.[36] All 13 SNPs at the *C2-BF-RDBP-SKIV2L-STK19* locus were genotyped, with a genotyping success rate and accuracy greater than 99%, as judged by random re-genotyping of 10% of the samples in the subject group.

Gene Expression

We used RT-PCR to detect the expression level of the *SKIV2L* gene in human cell line D407 (established from a primary culture of human RPE cells) and human tissues, including the retinas, RPE, heart, and kidney. These tissues were obtained from a deceased 55-year-old Han Chinese male. The total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA) and purified by chloroform extraction and isopropanol precipitation. Reverse transcription was performed with a reverse-transcription kit (Invitrogen). The forward and reverse primers (5′-GACAGAGACCCAGCACATGA-3′ and 5′-TCATCTGGA

Table 2. Association Between AMD and Genetic Variants at *C2-BF-RDBP-SKIV2L-STK19* Gene Region in a Han Chinese Population

SNP	Chr6 Position	Gene	Location	Minor Allele	MAF		P_HWE‖		Allelic *P*†	Corrected *P*‡	OR (95% CI)§
					Case	Control	Case	Control			
rs2734335	32001923	*C2*	Intron	G	0.370	0.360	0.656	0.139	0.6185	1	1.04 (0.89–1.23)
rs9332739*	32011783	*C2*	E318D	C	0.010	0.025	0.829	0.425	0.0112	0.1456	0.40 (0.19–0.82)
rs547154*	32018917	*C2*	Intron	T	0.041	0.055	0.782	0.958	0.1258	1	0.72 (0.50–1.07)
rs4151667*	32022003	*BF*	L9H	A	0.011	0.027	0.810	0.380	0.0094	0.1222	0.43 (0.22–0.84)
rs641153*	32022159	*BF*	R32Q	A	0.038	0.052	0.649	0.845	0.1115	1	0.73 (0.49–1.09)
rs760070*	32027935	*RDBP*	3′ UTR	C	0.031	0.048	0.384	0.633	0.0422	0.5486	0.66 (0.44–0.99)
rs3880457	32028198	*RDBP*	Intron	C	0.022	0.038	0.093	0.198	0.0302	0.3926	0.59 (0.37–0.96)
rs9501161	32032306	*RDBP*	Intron	A	0.037	0.056	0.417	0.059	0.0297	0.3861	0.65 (0.44–0.97)
rs437179	32036993	*SKIV2L*	M214L	A	0.308	0.315	0.009	0.224	0.813	1	0.98 (0.83–1.16)
rs429608*	32038441	*SKIV2L*	Intron	A	0.050	0.089	0.359	0.224	0.000376	0.00489	0.55 (0.40–0.77)
rs410851	32044647	*SKIV2L*	Y1067Y	T	0.347	0.317	0.508	0.120	0.0969	1	1.15 (0.97–1.37)
rs2075702	32045490	*SKIV2L*	3′ UTR	C	0.021	0.036	0.070	0.758	0.0376	0.4888	0.60 (0.37–0.98)
rs474534	32,046,086	STK19	5′ UTR	G	0.107	0.120	0.977	0.603	0.3729	1	0.88 (0.69–1.14)

UTR, untranslated region.

* These six SNPs were reported to be associated with AMD in previous studies, the remaining seven SNPs were haplotype tagging SNPs.

† Allelic *P* value has been adjusted for age and sex.

‡ Corrected *P* = Allelic *P* × 13 (the number of genotyped SNPs).

§ ORs (95% CI) were determined by the χ^2 test, cases versus controls.

‖ P_HWE was the *P* value of the Hardy-Weinberg equilibrium (HWE) testing.

Table 3. Conditional Analysis of the *C2-BF-RDBP-SKIV2L-STK19* Locus

SNP	Chr6	Gene	Location	Minor Allele	Conditional on *C2* rs9332739		Conditional on *BF*rs4151667		Conditional on *SKIV2L* rs429608	
					*P**	OR*	*P**	OR*	*P**	OR*
rs2734335	32001923	*C2*	Intron	G	0.511	0.92	0.387	1.17	0.897	0.98
rs9332739	32011783	*C2*	E318D	C	NA	NA	0.659	0.72	0.169	0.58
rs547154	32018917	*C2*	Intron	T	0.160	0.75	0.948	0.93	0.149	1.50
rs4151667	32022003	*BF*	L9H	A	0.361	0.52	NA	NA	0.167	0.59
rs641153	32022159	*BF*	R32Q	A	0.984	1.02	0.984	0.98	0.604	0.44
rs760070	32027935	*RDBP*	3′ UTR	C	0.040	0.62	0.928	1.11	0.567	1.20
rs3880457	32028198	*RDBP*	Intron	C	0.592	0.51	0.605	1.91	0.547	0.47
rs9501161	32032306	*RDBP*	Intron	A	0.023	0.62	0.022	0.62	0.017	0.61
rs437179	32036993	*SKIV2L*	M214L	A	0.017	0.63	0.017	1.59	0.024	0.64
rs429608	32038441	*SKIV2L*	Intron	A	0.00769	0.58	0.00431	0.59	NA	NA
rs410851	32044647	*SKIV2L*	Y1067Y	T	0.955	1.01	0.065	1.42	0.841	0.98
rs2075702	32045490	*SKIV2L*	3′ UTR	C	0.017	0.52	0.342	2.08	0.031	0.55
rs474534	32046086	*STK19*	5′ UTR	G	0.884	0.92	0.912	1.06	0.960	0.97

* The results of association testing of the *C2-BF-RDBP-SKIV2L-STK19* locus when the allelic dosage of rs9332739 (*C2*), rs4151667 (*BF*), or rs429608 (*SKIV2L*) was included in the regression model.

TAGGGCACCTC-3′, respectively) were used for *SKIV2L* PCR and gave a 246-bp product. The housekeeping gene *β-actin*, forward primer 5′-TGACGTGGACATCCGCAAAG-3′, and reverse primer 5′-CTGGAAGGTGGACAGCGAGG-3′, was used as an internal control and produced a 205-bp product. All RT-PCR products were confirmed by direct sequencing.

Immunohistochemistry

For immunohistochemistry, eyes were harvested from C57Bl/6J mice (The Jackson Laboratory, Bar Harbor, ME) at 2 months of age, fixed overnight in 4% paraformaldehyde in PBS, and then washed three times in PBS to remove trace amounts of formaldehyde. Eyes were then processed and embedded in paraffin for sectioning at 7 μm. Sections were stained with a rabbit anti-*SKIV2L* antibody (1:200; Proteintech, Chicago, IL) and visualized with rhodamine red–conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratory, Inc., West Grove, PA). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO). Sections were analyzed and imaged on a Nikon microscope (Nikon, Tokyo, Japan).

Statistical Analysis

We tested the Hardy-Weinberg equilibrium (HWE) for each SNP using the χ^2 test with df = 1. The genetic association analysis was carried out by constructing 2×2 tables of the allele counts and 2×3 tables of the genotype counts for each SNP in all cases and controls. Subsequently, Pearson χ^2 statistics were calculated and *P* values were computed by comparing the statistic to a χ^2 distribution with 1 or 2 degrees of freedom for the allelic and genotypic tests. The odds ratios (ORs) of the alleles and genotypes were estimated by using the χ^2 test. All statistical analyses were performed using SPSS (version 10.0) software (IBM SPSS Statistics, Chicago, IL). Haplotype analysis was carried out with Haploview 4.2 software (Daly Lab at the Broad Institute, Cambridge, MA).[37]

Conditional Analysis of *C2-BF-RDBP-SKIV2L-STK19* Locus

Conditional analyses of the *C2-BF-RDBP-SKIV2L-STK19* locus were completed at rs2734335 (*C2*), rs9332739 (*C2*), rs547154 (*C2*), rs4151667 (*BF*), rs641153 (*BF*), and rs429608 (*SKIV2L*) in the AMD cases and controls. The allelic dosage of each SNP was individually included as an additional covariate in the logistic regression model, along with age and sex.

Results

All 13 SNPs were successfully genotyped. The distribution of all 13 SNPs was within HWE ($P > 0.05$, Table 2). Pearson χ^2 statistics were used to investigate the association between the SNPs and AMD. We found that the allelic *P* values of seven SNPs were less than 0.05 before multiple testing (Table 2). However, only the *P* value of rs429608 still showed significant association with AMD after the Bonferroni correction ($P = 0.00489$), with an OR of 0.55 (95% confidence interval [CI] = 0.40–0.77) (Table 2). None of the other 12 SNPs showed significant association with AMD after the Bonferroni correction ($P > 0.05$, Table 2).

To verify our results, we took the three most significant association SNPs (*C2* rs9332739, *BF* rs4151667, and *SKIV2L* rs429608) and sequentially conditioned on the minor allele of each variant. Results of the conditional analysis showed that rs429608 was still the most significantly associated SNP with AMD in the *C2-BF-RDBP-SKIV2L-STK19* locus (Table 3). No haplotype was generated from these 13 SNPs genotyped through Haploview analysis; this is similar to the data of HapMap CHB database (HapMap Public Release #27 on Genome Browser) (Fig. 1).

We further validated the association between rs429608 of the *SKIV2L* gene and AMD by using different genetic models. The genotype distribution of rs429608 is shown in Table 4. The estimated AMD risk was significantly lower in subjects carrying rs429608AA or rs429608AG than in those carrying rs429608GG, indicating a dominant model for the A-allele. Thus, the dominant model was applied to analyze the variant's association with AMD. The frequency of the rs429608 AA+AG genotype was significantly lower in the AMD patients than in the controls (9.5% vs. 16.6%, $P = 0.000343$, OR [95% CI] = 0.53 [0.38–0.76], Table 4), and the AG genotype resulted in a significantly protective effect of AMD ($P = 0.000526$, OR [95% CI] = 0.54 [0.38–0.77], Table 4).

As shown in Figure 2, the *SKIV2L* gene was expressed in human RPE and D407 cells, as well as in the human heart and kidneys; it was also expressed in human retina. We then checked the expression of *SKIV2L* in various human tissues

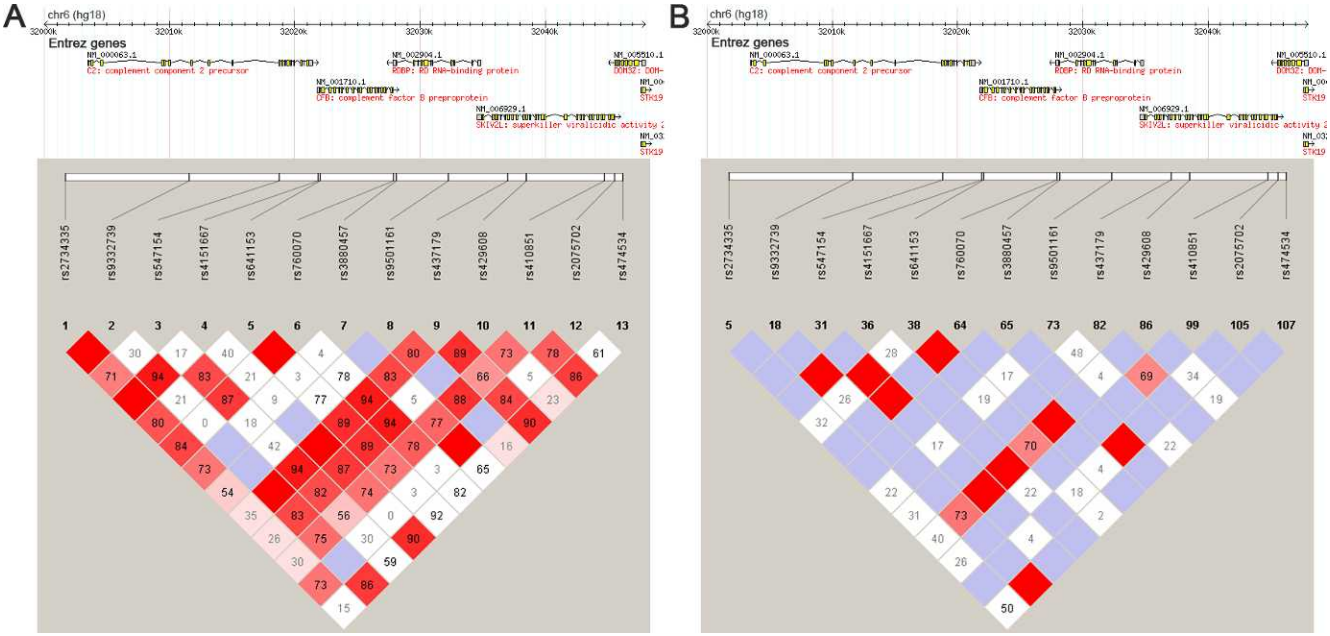

Figure 1. LD block structure across the *C2-BF-RDBP-SKIV2L-STK19* gene region (r^2 values shown). *White*, $r^2 = 0$; *gray*, $0 < r^2 < 1$; *black*, $r^2 = 1$. (**A**) This LD block was generated using the data from all subjects in the present study. The LD block structure was examined using the program Haploview (version 4.2). The D' values and r^2 values for all pairs of SNPs were calculated, and the haplotype blocks were estimated using the program Haploview. (**B**) HapMap CHB data (downloaded) of these 13 SNPs at the *C2-BF-RDBP-SKIV2L-STK19* gene region. The physical position of each SNP is shown in the *upper diagram*.

and cell lines via NCBI Geoprofiles (http://www.ncbi.nlm.nih.gov/geo/). We found that our results in this study were very similar to the data in the NCBI Geoprofiles. Further detection by immunohistochemistry showed that *SKIV2L* was expressed in the mouse retinal outer segment, outer nuclear layer, and RPE layer (Fig. 3).

Discussion

Based on the fact that the genetic variants in *CFH* are significantly and robustly associated with AMD, Gold et al.[24] investigated whether genetic variants of *BF* and *C2*, which are on the same pathway as *CFH*, are associated with AMD. They found that the *BF* variants rs4151667 (L9H) and rs641153 (R32Q), and *C2* variants rs9332739 (E318D) and rs547154 (intron 10) were significantly associated with AMD in 900 individuals with AMD and approximately 400 controls. This finding was then successfully replicated in different populations[28,38] However, we did not find a significant association between these four SNPs and AMD in a Han Chinese population.[29] Consistent with our findings, Kim et al.[31] also failed to find a significant association between these four SNPs and AMD in a Korean population. Although Nakata et al.[39] found that rs547154 in the *C2* gene and rs541862 in the *BF* gene are significantly associated with CNV AMD (*P* values of 0.018 and 0.016, respectively) in a Japanese population, this association would not be significant after correction for multiple testing.

The presence or absence of an allelic association often depends on the allele frequency in the population. One of the reasons for the inability to replicate the association between these genetic variants in *C2/BF* and AMD in East Asian populations might be that the minor allele frequencies of these SNPs are too low (less than 0.05, except rs641153, which is 0.052, Table 2). This suggests that other unidentified SNPs in *C2* and *BF* may be associated with AMD in the East Asian population. It is also possible that genetic variants in neighboring genes may be associated with AMD at this locus. This is supported by a recent study by McKay et al.,[33] who genotyped 18 haplotype-tagging SNPs at this *C2/BF* locus in 318 patients with wet AMD and 243 age-matched controls in Caucasians. They identified that rs438999 (R151Q) in the *SKIV2L* gene, which is in the same linkage disequilibrium (LD) block as *BF* rs641153 (R32Q) ($r^2 = 0.95$), is significantly

Table 4. The SNP of rs429608 in the *SKIV2L* Gene Was Associated With AMD in This Study

Group	Genotype, *n* (%)			Model	Crude OR, 95% CI	Adjusted OR, 95% CI	Adjusted *P*
	AA	AG	GG				
Control	11 (1.1)	156 (15.5)	837 (83.4)		1	1	
AMD	2 (0.5)	40 (9.0)	402 (90.5)	Additive 1	0.53 (0.37–0.77)	0.54 (0.38–0.77)	0.000526
				Additive 2	0.38 (0.08–1.71)	0.46 (0.10–2.15)	0.3149
				Dominant	0.52 (0.36–0.75)	0.53 (0.38–0.76)	0.000343
				Recessive	0.41 (0.09–1.85)	0.50 (0.11–2.33)	0.3704

Crude ORs (95% CI) were determined by the χ^2 test, cases versus controls. Adjusted ORs (95% CI) and adjusted *P* values were obtained by adjusting for age and sex. Genotype (AA/AG/GG) analyses were conducted for the dominant model (AG+AA compared with GG), additive model 1 (AG compared with GG), additive model 2 (AA compared with GG), and the recessive model (AA compared with AG+GG).

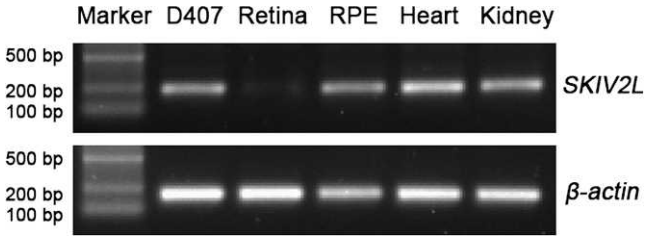

FIGURE 2. Expression of the *SKIV2L* gene in human cells and tissues. RT-PCR analyses of *SKIV2L* gene expression in the human retina, RPE, heart, kidney, and D407 (RPE) cells with 246 bp of products. We used β-actin as an internal control for cDNA quantification.

associated with CNV AMD ($P = 0.0004$). They concluded that variations within *SKIV2L* might exert a functional effect in AMD. Furthermore, Kopplin et al.[34] performed a genome-wide association study (GWAS) in 1896 cases of AMD and 1866 controls and observed a protective effect of an intronic SNP, rs429608 ($P = 5.3 \times 10^{-15}$), to AMD in the *SKIV2L* gene. In addition, Lee et al.[40] and Kondo et al.[41] investigated the association between the *C2/BF* locus and PCV, which is one type of wet AMD (wet AMD includes CNV and PCV); they could not find a significant association between the previously reported SNPs and PCV in Singapore Chinese and Japanese individuals. On the other hand, Kondo et al.[41] found that two SNPs (rs3880457 in *RDBP* and rs2075702 in *SKIV2L*), which are in the same LD block ($r^2 = 1.0$), are significantly associated with PCV in a Japanese population.

In our study, we confirmed that rs429608 in *SKIV2L*, but none of the SNPs in *C2* and *BF*, is significantly associated with CNV AMD after correction of multiple testing. We further evaluated the association between rs429608 and AMD by using the dominant model. We found that the frequency of the rs429608 AA+AG genotype was significantly lower in the AMD patients than in the controls (9.5% vs. 16.6%, $P = 0.000343$, OR [95% CI] = 0.53 [0.38, 0.76], Table 4), and the AG genotype showed a significantly protective effect for AMD ($P = 0.000526$, OR [95% CI] = 0.54 [0.38, 0.77], Table 4). A large GWA study of AMD was recently carried out in the Japanese population,[42] with a total of 1536 AMD cases and 18,894 controls. Two new susceptibility loci (8p21 and 4q12) were identified. In addition, as shown in Supplementary Table 2 of this large GWA study,[42] a similar trend can be found (rs429608, minor allele frequency [MAF] is 0.070 in cases and 0.113 in controls, $P = 2.24 \times 10^{-7}$) compared with our results (rs429608, MAF is 0.050 in cases and 0.089 in controls, $P = 3.76 \times 10^{-4}$, Table 2). The data strongly support our results. However, for some unknown reason, the Japanese group did not focus on this finding and instead emphasized two novel loci (8p21 and 4q12). Actually, rs429608 in the *SKIV2L* gene was more significantly associated with AMD than SNPs in the *C2* and *BF* genes in the Japanese GWAS article, which also suggested an important role for *SKIV2L* in the development of AMD in the Japanese. Taking our results and these previous studies together, it is more likely that *SKIV2L* is the AMD-protective gene at this AMD-associated locus, rather than *C2/BF*.

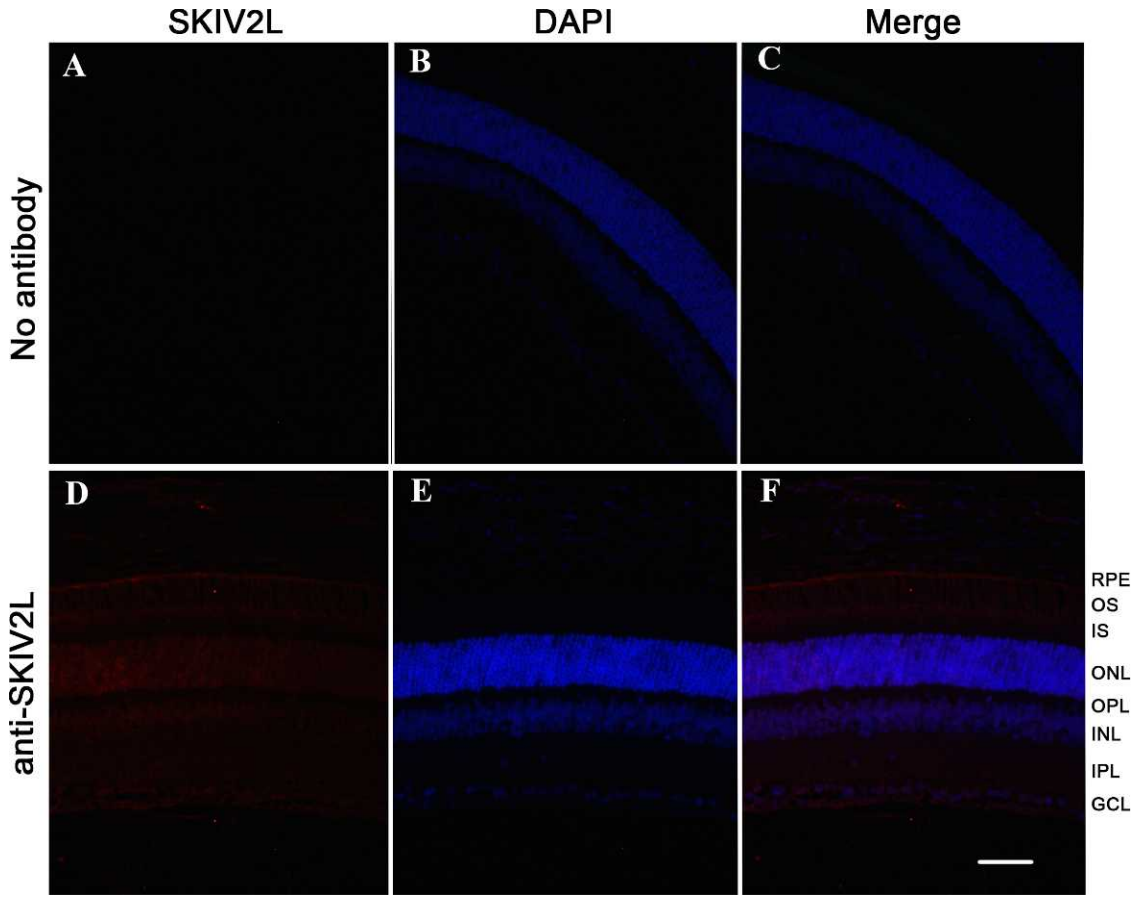

FIGURE 3. SKIV2L is expressed in mouse retinal OS and RPE. (**A–C**) Sections stained with only secondary antibody. No SKIV2L can be detected. (**D–F**) Sections stained with anti-SKIV2L antibody. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer. Animals were killed at 2 months of age. The *scale bar* is 50 μm.

SKIV2L, a DEAD box protein that is characterized by the conserved motif Asp-Glu-Ala-Asp (DEAD), is a putative RNA helicase and is expressed in the human RPE (Fig. 2). Loss of RPE is a main feature of AMD. Therefore, it is possible that *SKIV2L* plays an important role in the development of AMD.

Because we did not analyze the whole sequence of the AMD-associated block on chromosome 6 at this stage, we could not completely exclude the role of *C2* and *BF* in the development of AMD because *C2* and *BF* are functionally related to the CFH pathway and the pathogenesis of AMD. The rs429608 is located in the intronic region of *SKIV2L*. We do not understand how this SNP affects the function of SKIV2L, and other functional SNPs in *SKIV2L* have not yet been identified. Biological function studies of SKIV2L may provide further insights into the pathogenesis of AMD and may make this conclusion solid.

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