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Localization of age-related macular degeneration-associated ARMS2 in cytosol, not mitochondria

Gaofeng Wang^{1,*}, Kylee L. Spencer², Brenda L. Court¹, Lana M. Olson², William K. Scott¹, Jonathan L. Haines², and Margaret A. Pericak-Vance¹

¹Miami Institute for Human Genomics, University of Miami, Miami, FL 33136

²Center for Human Genetics Research, Vanderbilt University, Nashville, TN 37232

Abstract

PURPOSE—To analyze the relationship between ARMS2 and HTRA1 in the association with age-related macular degeneration (AMD) in an independent case-control dataset, and to investigate the subcellular localization of the ARMS2 protein in an *in vitro* system.

METHOD—Two SNPs in ARMS2 and HTRA1 were genotyped in 685 cases and 269 controls by Taqman Assay. Allelic association was tested by a χ^2 test. A likelihood ratio test (LRT) of full vs. reduced models was utilized to analyze the interaction between ARMS2 and smoking and HTRA1 and smoking, after adjusting for CFH and age. Immunofluorescence and immunoblot were applied to localize ARMS2 in retinal epithelial ARPE-19 cells and COS7 cell transfected by ARMS2 constructs.

RESULT—Both significantly associated SNP rs10490924 and rs11200638 ($P < 0.0001$) are in strong linkage disequilibrium (LD) ($D' = 0.97$, $r^2 = 0.93$) that generates virtually identical association test and odds ratios. In separate logistic regression models the interaction effect for both smoking with ARMS2 and with HTRA1 was not statistically significant. Immunofluorescence and immunoblot show that both endogenous and exogenous ARMS2 are mainly distributed in the cytosol, not the mitochondria. Comparing to wild type, ARMS2 A69S is more likely to be associated with cytoskeleton in COS7 cells.

CONCLUSIONS—The significant associations in ARMS2 and HTRA1 are with polymorphisms in strong LD that confer virtually identical risks, preventing differentiation at the statistical level. We found that ARMS2 was mainly distributed in the cytosol, not in mitochondrial outer membrane as previously reported, suggesting that ARMS2 may not confer risk to AMD through the mitochondrial pathway.

INTRODUCTION

Age-related macular degeneration (AMD, MIM 603075) affects the central part of the human retina and causes a progressive degeneration in detailed central vision. Currently, AMD is the leading cause of visual impairment and blindness in developed countries. Epidemiologically, AMD is a common complex disorder. Present studies suggest that both environmental and genetic factors contribute to AMD [1,2]. Of the many postulated environmental factors, cigarette smoking has the strongest influence on risk for AMD [3,4]. Genetically, among a list of proposed chromosomal regions, the loci at 1q32 and 10q26 have

*Address for correspondence: Dr. Gaofeng Wang, Miami Institute for Human Genomics, University of Miami Miller School of Medicine, Miami, FL 33136, gwang@med.miami.edu, Telephone: (305) 243- 5434, Fax: (305) 243- 2396.

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been repeatedly and consistently linked to the disease in multiple studies [5–9]. Subsequently, the Y402H variant in the *CFH* (complement factor H, MIM 134370) gene, located on chromosome 1q32, was discovered as the first major AMD susceptibility allele [10–15].

In contrast it has been difficult to identify with certainty the susceptibility variation(s) responsible for linkage and association to the locus on chromosome 10q26. There are three genes located in this region, *PLEKHA1* (Pleckstrin homology domain containing, family A, member 1, MIM 607772), *ARMS2* (age-related macular degeneration susceptibility 2, MIM 611313) and *HTRA1* (HtrA serine peptidase 1, MIM 602194). Each of these three genes, especially the latter two, have been suggested the susceptibility gene [16–19]. Unfortunately, the polymorphisms in *ARMS2* (rs10490924; nonsynonymous A69S change) and *HTRA1* (rs11200638; promoter polymorphism) associated with AMD are in such strong linkage disequilibrium that their effects are indistinguishable using statistical analysis [16–19]. Studies, including ours, have demonstrated a statistical interaction between smoking and chromosomal 10q26 genes, especially *ARMS2*, in the association with AMD, suggesting *ARMS2* as the most likely candidate for the second major AMD susceptibility gene [7,20,21].

Recently, Kanda et al reported that SNP rs10490924 (*ARMS2* A69S) alone could explain the bulk of the association between the chromosomal 10q26 region and AMD. *In vitro* experiments showed that *ARMS2* localized to the mitochondrial outer membrane. Based on these observations, they suggested that *ARMS2* is the AMD susceptibility gene and may confer the risk through mitochondrial pathway [22].

To extend the findings by Kanda et al., we repeated their case-control analysis in our independent data set and attempted to replicate their *in vitro* experiment findings.

MATERIAL AND METHODS

Samples

The independent case-control sample set contains 685 AMD cases and 269 unrelated controls, of which 456 cases and 234 controls have smoking data as shown in table 1 and described in detail elsewhere [23]. All participants are non-Hispanic Caucasians. All patients and controls received an eye examination and had stereoscopic fundus photographs graded according to a modified version of the age-related eye disease study (AREDS) grading system as described previously [24]. Briefly, grades 1 and 2 represent controls. Grade 1 controls have no evidence of drusen or small non-extensive drusen without pigmentary abnormalities, while grade 2 controls may show signs of extensive small drusen, non-extensive intermediate drusen and/or pigmentary abnormalities. Grade 3 AMD cases have extensive intermediate drusen or large, soft drusen with or without drusenoid retinal pigment epithelial detachment. Grade 4 AMD cases exhibit geographic atrophy and grade 5 individuals have exudative AMD, which includes non-drusenoid retinal pigment epithelial detachment, choroidal neovascularization, and subretinal hemorrhage or disciform scarring. Individuals were classified according to status in the more severely affected eye. Approval for the study was obtained from the appropriate institutional review boards at Vanderbilt University Medical Center, Duke University Medical Center, and the University of Miami Miller School of Medicine; all study participants gave informed consent, and this research adhered to the tenets of the Declaration of Helsinki.

DNA extraction and SNP genotyping

Genomic DNA was extracted from whole blood using the PureGene system (Gentra Systems). Primers and probes were designed using the Primer Express 2.0 program (Applied

Biosystems). Two SNPs including rs10490924 and rs11200638 were genotyped using the Taqman Assay. The fluorescence generated during the PCR amplification was detected using the ABI Prism 7900HT sequence detection system and was analyzed with SDS software (Applied Biosystems). Quality control samples were duplicated within and between plates, and we required that 95% of individuals assayed receive a genotype for SNPs to be used in further analyses.

Statistical analysis

We verified that all SNPs were in Hardy–Weinberg equilibrium (HWE) and examined the HWE and LD in both the overall dataset and separately in cases and controls, using Haploview software (data not shown) [25]. We assessed the association of each SNP with AMD using the $2 \times 2 \chi^2$ test for allelic association, and estimated age-adjusted odds ratios using logistic regression. We used conditional analyses to test for the effect of ARMS2 A69S in rs11200638 carriers and vice versa. The effects of ARMS2 A69S and HTRA1 rs11200638 were estimated in separate logistic regression models after controlling for age, smoking status, and the Y402H variant in CFH, assuming an additive genetic model for each locus. Smokers (those who had smoked at least 100 cigarettes) were coded as ‘1’ and non-smokers (those who had smoked fewer than 100 cigarettes over their lifetime) were coded as ‘0’. We tested for interactions between smoking and ARMS2 A69S or HTRA1 rs11200638 by comparing full and reduced logistic regression models with a likelihood ratio statistic (LRT, twice the difference in the deviance of the full compared with reduced logistic regression models) and determined significance by comparing the LRT with a χ^2 distribution with 1 degree of freedom. All case–control analyses were performed using Intercooled Stata 9.1 (StataCorp LP).

Polyclonal antibody generation

One polyclonal antibody was custom produced against synthetic peptides of ARMS2 by Bethyl Laboratories (Montgomery, TX). The targeted C-terminal peptide is CSPAGTQRRFQQPQHHLT (amino acids 82–98). Rabbits were immunized at multiple subcutaneous sites multiple times. Antibodies were purified using immunoaffinity columns. Endogenous ARMS2 protein was detected by immunofluorescence in ARPE-19 cells with the primary antibody against ARMS2.

Plasmids constructs and mutagenesis

ARMS2 expression constructs were made from RT-PCR and TA cloning into GFP or His-tag vectors. Human retinal RNA was reverse transcribed to cDNA. The fresh PCR products were cloned into pcDNA3.1-NT-GFP, pcDNA3.1-CT-GFP and pcDNA3.1-His according to manufacturer’s instructions (Invitrogen). The PCR were applied by using the following two pair primers:

NT-GFP-forward: ATGCTGCGCCTATACCCAGG

NT-GFP-reverse: TCACCTTGCTGCAGTGTGGATGAT

CT-GFP-forward: ATGCTGCGCCTATACCCAGG

CT-GFP-reverse: TTGCTGCAGTGTGGATGATAG

HIS-Tag-forward: ATGCTGCGCCTATACCCAGG

HIS-Tag-reverse: CCTTGCTGCAGTGTGGATGATAGAC

The PCR product was separated in agarose gel and extracted, purified and cloned with TA cloning Kit (Invitrogen). Finally, the pcDNA3.1-CT-GFP-A69S construct was generated using the Quickchange XL site-directed mutagenesis kit (Stratagene) by using forward

mutagenic primer (5'-CACACTCCATGATCCCAGCTTCTAAAATCCACACTGAGCTCTGC-3') and a complementary reverse mutagenic primer (5'-GCAGAGCTCAGTGTGGATTTTAGAAGCTGGGATCATGGAGTGTG-3'). All the resulting constructs were verified by sequencing.

Cell Culture and Transfection

Human retinal epithelial ARPE-19 and Green monkey kidney epithelial COS7 cells were obtained from American Type Culture Collection. ARPE-19 cells were maintained in DMEM-F12 medium and COS7 cells were maintained in DMEM. Both culture media contained 10% FBS. COS7 Cells were cultured in 6 well plates with cover slips and transiently transfected 2 μ g pcDNA3.1 ARMS2 constructs by using the appropriate amount of Lipofectamine LTX according to the manufacturer's instructions (Invitrogen).

Immunofluorescence

ARPE-19 cells were cultured in 6 well plates with cover slips. After seeding 24 hours, cells were washed by PBS twice and fixed by 4% paraformaldehyde for 15min at room temperature, then incubated with 10% normal donkey serum (Jackson ImmunoResearch, West Grove, PA) in PBS. Cells were subsequently incubated for 2 hours at room temperature with the ARMS2 antibody (1:200) in PBS. After washing with PBS, cells were then incubated with FITC-conjugated donkey anti-rabbit IgG (1:200; Jackson ImmunoResearch) for another 2 hours. ARPE-19 cells were either co-stained with mitoTracker Red, Rhodamine phalloidin (Invitrogen), or double immunostained with anti-tubulin, anti-calnexin, anti-golgin, and anti-LAMP1 antibodies respectively. Cy3-conjugated donkey anti-mouse IgG (1:200; Jackson ImmunoResearch) was used as a second immunofluorescence.

After transfection for 24 hours, COS7 cells were either co-stained with mitoTracker Red, rhodamine, phalloidin or double immunostain with the antibodies mentioned above. Double fluorescence images were acquired with a Zeiss LSM510 confocal microscope.

Mitochondrial isolation and immunoblot

Mitochondria were extracted from ARPE-19 cells using MitoProfile benchtop mitochondria isolation kit (Mitoscience) according to the manufacturer's instruction. Briefly, ARPE-19 cells at 90% confluence were detached from 100mm dish and centrifuged at 1,000g for 3 minutes. Cells were re-suspended with Reagent A followed by homogenization. The homogenate was centrifuged at 1,000 g for 10 minutes. The pellet was added to Reagent B, homogenized, and spun down again. The combined supernatants were further centrifuged at 12,000 g for 15 minutes. The resulting supernatant was collected as cytosol and the pellet was removed and dissolved in reagent C as mitochondria. Cytosol and mitochondrial proteins were resolved on 4–20% SDS-polyacrylamide gels (Bio-Rad), transferred to nitrocellulose membranes, and immunoblotted with anti-ARMS2, anti-porin and anti- β -actin antibodies. Proteins were visualized by using chemiluminescence.

RESULTS

ARMS2 or HTRA1: statistically indistinguishable association with AMD

Both rs10490924 and rs11200638 were strongly associated with AMD ($p < 0.0001$, Table 2). The strong linkage disequilibrium (LD) between these two SNPs ($D' = 0.97$, $r^2 = 0.93$) makes it impossible to determine which variant is the biological relevant one by statistical modeling, as both variants produce nearly identical association test statistics and odds ratios (Table 2). Using conditional analyses to evaluate models of these two SNPs, the significant

association of one SNP is lost after controlling for the effect of the other association, and vice versa (data not shown). As smoking has been shown to modify the association of 10q26 genes with AMD by our group and others [7,20,21], we tested for interaction between rs10490924 and rs11200638 and smoking in separate logistic regression models, adjusting for age and CFH genotype. Not unexpectedly due to the strong LD, likelihood ratio test (LRT) statistics were again similar for both SNPs (rs10490924 LRT=2.89 and rs11200638 LRT=2.71, but in this updated analysis with larger sample size the interaction effect with smoking was not statistically significant (rs10490924-smoking $p=0.09$ and rs11200638-smoking $p=0.10$).

ARMS2 is localized in cytosol, not mitochondria

To test whether ARMS2 is imported into the mitochondria, we generated a polyclonal antibody targeting the C-terminus (82–98 amino acid sequence). Immunofluorescence showed that endogenous ARMS2 is not co-localized with mitochondrial marker at all in epithelial retina ARPE-19 cells. Experiments using pre-immune serum and blocking peptide confirmed the specificity of this immunostaining (Fig 1). By overexpressing GFP-tagged and HIS-tagged ARMS2 constructs, we further studied exogenous ARMS2 localization in COS7 cells which share most common cell biological characteristics with COS1 cells (www.atcc.org) used by Kanda et al [22]. Confocal images clearly showed no co-localization of both endogenous and exogenous ARMS with the mitochondrial marker (Fig. 2). Immunoblot further confirmed that ARMS2 was present only in the cytosolic but not the mitochondrial cellular fraction as shown in Figure 3.

To confirm the subcellular targeting of ARMS2, we applied *in silico* analyses. Characteristic features of the mitochondrial targeting signals are several positively charged amino acid residues with a few intervening uncharged amino acids at the N-terminus of mitochondrial protein precursors [27]. ARMS2 lacks charged amino acids at both the N-terminus (1 out of first 10 aa sequence) and C-terminus (2 out of last 10 aa sequence). We used the program WoLF PSORT (<http://wolfsort.org/>) to predict the signal targeting activities of the ARMS2. The analysis suggests a multi-subcellular localization of ARMS2, including cytosol and nucleus, but not mitochondria (Table 3). Another bioinformatics tool MitoProt II (<http://ihg2.helmholtz-muenchen.de/ihg/mitoprot.html>) predicts the probability of ARMS2 for mitochondrial targeting is 0.0185, while probability for the positive control mitofusin 2 is 0.7226 and the negative control EGF is 0.0063.

To determine ARMS2 subcellular targeting, we then co-labeled ARPE-19 and COS7 cells with markers of the nucleus, microtubules, actin, Golgi apparatus, endoplasmic reticulum and lysosome. These double immunofluorescences demonstrated that most ARMS2 existed in the cytosol of the perinuclear region. While a small portion of ARMS2 displayed in the nucleus, there is no co-localization within the cellular organelles investigated or the cell cytoskeleton system (Fig. 1 & 2). And immunostaining of the transfected COS7 cells shows the specificity of ARMS2 antibody which staining is largely co-localized with GFP or His-tag signals. All these results suggest that ARMS2 does not localize to the mitochondria, but is instead mainly a cytosolic protein.

ARMS2 A69S is associated with cell skeleton

To explore the biological effects of ARMS2 A69S, we made GFP-tagged-ARMS2-A69S constructs. After transfection in COS7 cells, ARMS2 containing A69S replacement is not localized in mitochondria (Fig 4) or any other cellular organelles (data not shown). Interestingly, some ARMS2 A69S seems to be co-localized and distributed along the cytoplasmic skeleton including microtubule and actin (stained by phalloidin, Fig 4). The biological effect of this association between ARMS2 A69S and microtubule/actin is unclear.

DISCUSSION

Linkage of AMD to chromosome 10q26 has been confirmed in multiple studies [5–9]. Each of three genes, *PLEKHA1*, *HTRA1* and *ARMS2* in this region has been suggested as a susceptibility gene for AMD by several association analyses. Interestingly, two independent studies identified SNP rs11200638, located at a proposed promoter region for *HTRA1*, as the most likely AMD susceptibility variant. In these studies, the risk allele of rs11200638 was correlated with higher *HTRA1* expression levels in peripheral lymphocytes [18,19]. Contrary to these findings, Kanda et al. found that rs11200638 had no significant impact on *HTRA1* promoter activity in cell lines and retina tissues. By evaluating 45 tag SNPs spanning *PLEKHA1*, *ARMS2* and *HTRA1* gene in 466 cases and 280 controls, they reported that rs10490924 could explain the bulk of the association between the 10q26 region and AMD, whereas rs11200638 could not. They concluded that it is *ARMS2*, not *HTRA1*, is the most likely susceptibility gene for AMD [22].

From our independent case-control dataset, both rs10490924 and rs11200638 are strongly associated with AMD, while also being in strong linkage disequilibrium. We cannot confirm that rs10490924 alone is directly responsible for the association between the 10q26 region and AMD in our statistical analyses. The contribution of these two SNPs in the association with AMD is statistical indistinguishable. We also tried to determine the susceptibility gene from their interaction with environmental risk factors. In our previous study, smoking has been shown to modify the association of *ARMS2* gene with AMD while SNP rs11200638 of *HTRA1* was not included in that dataset [20]. In this updated analysis with larger sample size the interaction effect with smoking was not statistically significant for both SNPs. We believe that the available statistical methods cannot separate the role of *ARMS2* or *HTRA1* in AMD as indicated in previous study [22]. Future biological function studies on *ARMS2* and *HTRA1* may provide more evidence to determine their status in AMD.

The concept of *ARMS2* localizing at mitochondria, as reported by Kanda et al. is very attractive for this degenerative disease. However, by immunofluorescence and immunoblot, we found that endogenous *ARMS2* is not localized in mitochondria of retina epithelial ARPE-19 cells. Furthermore, exogenous *ARMS2* is not localized in mitochondria of COS7 either, after the cells being transfected with N-terminal or C-terminal GFP-tagged or C-terminal-HIS-tagged *ARMS2* constructs. In our experimental system, most of *ARMS2* is clearly localized in cytosol, and a small portion in nucleus. No mitochondrial *ARMS2* were detected. The co-localization image of *ARMS2* with mitochondrial marker in human retina reported by Fritsche et al is not convincing due to the low resolution and low magnification [28]. In our experimental system, we are unable to replicate the mitochondria targeting of *ARMS2* in COS cell and retina epithelium reported before [22,28]. The reason for this conflict probably lies in the difference between the *ARMS2* fragmental peptides used in antibody preparations. However, the largely co-localized fluorescence of GFP/His-tag with our *ARMS2* antibody staining further confirmed the specificity and consistency of endogenous and exogenous *ARMS2* cytosolic localization in COS7 and ARPE-19 cells.

Furthermore, *in silico* analyses also showed very low probability of *ARMS2* importing to mitochondria. Interestingly, comparing to wild type, *ARMS2* A69S is more likely co-localized with cellular skeleton including microtubule and actin, suggesting the replacement of Alanine by Serine may gain of function to interact with cytoskeleton. The biological meaning for *ARMS2* A69S association with microtubule / actin is waiting for further studies to explain.

Polymorphisms in *ARMS2* and *HTRA1* are strongly associated with AMD while the strong LD in the genomic region prevents determining which gene really driving the association at

the statistical level. We cannot confirm the ARMS2 localized at mitochondrial outer membrane as previously reported. We suggest that the action of ARMS2 may not be through mitochondrial pathway to confer risk to AMD if ARMS2 is the true AMD gene in 10q26 region.

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Fig 1.

Immunostaining of ARMS2 in ARPE-19 cells. ARMS2 is not localized within the mitochondria shown by mitoTracker. Furthermore, ARMS2 is not co-localized with the markers for other subcellular organelles and cytoskeleton either. These include golgi apparatus (anti-golgin), endoplasmic reticulum (anti-calnexin), lysosome (anti-LAMP1), microtubule (anti- γ -tubulin), F-actin (phalloidin) and nucleus (DAPI). Most ARMS2 is mainly localized in the perinuclear region and no specific organelle is found to host ARMS2. Preimmune serum and blocking peptide attenuating anti-ARMS2 antibody show negative staining.

Fig 2.

Exogenous ARMS2 is not found in any specific organelles. COS7 cells were transfected with N-terminal or C-terminal GFP (green fluorescence protein) tagged, His-tagged ARMS2 constructs and GFP control construct. Most GFP-ARMS2 and His-ARMS2 are mainly localized in the perinuclear region, while GFP control is distributed uniformly within the cell. Co-immunostaining with markers of different cellular organelles shows that no specific organelle is found to host ARMS2. Immunostaining with ARMS2 antibody is largely co-localized with GFP or His-tag staining.

Fig 3. ARPE-19 cells were homogenized and mitochondria were separated from the cytosolic fraction by centrifugation. The ARMS2 antibody was probed against ARPE-19 cell lysate on a western blot and showed a band of the expected size only in the cytosol, not in mitochondrial fraction. The blot was reprobed with porin, which constitutes a mitochondrial membrane protein, and β -actin.

Fig 4.
ARMS2 A69S is co-localized with microtubule (anti-tubulin) and actin (stained by phalloidin) while it does not co-localize with mitochondria.

Table 1

Demographic, Clinical and Genetic Characteristics of Study Population

Variable	Cases	Controls
Number	456	234
Age of Exam (AOE $\pm$ sd)	75.9 $\pm$ 7.5	66.4 $\pm$ 8.1
Race, % Caucasian	100	100
Gender (% Female)	61.8	54.7
Smoking (% Ever Yes)	60.7	49.1
rs1061170, Y402H freq risk allele C %)	58.6	42.1

Note: The total independent case-control sample set contains 685 AMD cases and 269 unrelated controls, of which 456 cases and 234 controls have smoking data, and whose data is presented in the table.

Table 2

Association of ARMS2 rs10490924 and HTRA1 rs11200638 with AMD

SNP	Case MAF*	Control MAF	χ^2	p-value	Age-adjusted Odds Ratio	95% Confidence Interval
rs10490924	41.7	26.2	24.9	<0.0001	2.09	1.63 2.67
rs11200638	41.4	25.8	24.8	<0.0001	2.07	1.62 2.65

* MAF=minor allele frequency

Table 3

Prediction of distribution of ARMS2 into subcellular organelles

Subcellular organelles	% of ARMS2 distribution	
	Complete protein sequence used for prediction	N-terminal 45 amino acids sequence used for prediction
Cytosol	37%	23%
Nucleus	54%	31%
Cytosol-Nucleus	34%	10%
Secretory Vesicles	0%	7%
Mitochondria	0%	0%