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Analysis of the indel at the *ARMS2* 3'UTR in age-related macular degeneration

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Abstract

Controversy remains as to which gene at the chromosome 10q26 locus confers risk for age-related macular degeneration (AMD) and statistical genetic analysis is confounded by the strong linkage disequilibrium (LD) across the region. Functional analysis of related genetic variations could solve this puzzle. Recently Fritsche et al. reported that AMD is associated with unstable *ARMS2* transcripts possibly caused by a complex insertion/deletion (indel; consisting of a 443 bp deletion and an adjacent 54 bp insertion) in its 3'UTR (untranslated region). To validate this indel, we sequenced our samples. We found that this indel is even more complex and is composed of two side-by-side indels separated by 17 bp: (1) 9 bp deletion with 10bp insertion; (2) 417 bp deletion with 27 bp insertion. The indel is significantly associated with the risk of AMD, but is also in strong LD with the non-synonymous single nucleotide polymorphism (SNP) rs10490924 (A69S). We also found that *ARMS2* is expressed not only in placenta and retina but also in multiple human tissues. Using quantitative PCR, we found no correlation between the indel and *ARMS2* mRNA level in human retina and blood samples. The lack of functional effects of the 3'UTR indel, the amino acid substitution of rs10490924 (A69S) and strong LD between them suggest that A69S, not the indel is the variant that confers risk of AMD. To our knowledge, it is the first time it's been shown that *ARMS2* is widely expressed in human tissues. Conclusively, the indel at 3'UTR of *ARMS2* actually contains two side-by-side indels. The indels are associated with risk of AMD, but not correlated with *ARMS2* mRNA level.

Introduction

Age-related macular degeneration (AMD, MIM 603075) is currently the leading cause of irreversible visual loss in developed countries. AMD deprives affected patients of detailed central vision due to progressive degeneration in the central part of the human retina. Genetic factors confer a major risk for developing AMD along with environmental influences (Klein et al. 2004; Khan et al. 2006). Loci at chromosome 1q32 and 10q26 have been repeatedly and

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consistently linked to the disease in multiple studies (Majewski et al. 2003; Seddon et al. 2003; Weeks et al. 2004; Kenealy et al. 2004; Iyengar 2004) and represent the strongest genetic effects in AMD. The Y402H variant in the *CFH* (complement factor H, MIM 134370) gene on chromosome 1q32 was identified as the first major AMD susceptibility allele and has been widely confirmed (Haines et al. 2005; Hageman et al. 2005; Klein et al. 2005; Edwards et al. 2005; Zarepari et al. 2005; Conley et al. 2005).

Controversy remains, however, about which gene at the chromosome 10q26 locus confers risk for AMD. The most significant association signal is generated from the polymorphisms rs10490924 in *ARMS2* (age-related macular degeneration susceptibility 2, MIM 611313) and rs11200638 in *HTRA1* (HtrA serine peptidase 1, MIM 602194). Due to the strong linkage disequilibrium (LD) across this region, statistical genetic analysis is limited in distinguishing the effects of either *ARMS2* or *HTRA1* (Jakobsdottir et al. 2005; Rivera et al. 2005; Dewan et al. 2006; Yang et al. 2006). Functional analysis of the related genetic variations in the genes *ARMS2* and *HTRA1* can be helpful in solving this puzzle. The rs11200638 risk allele located in the promoter of the gene *HTRA1* was reportedly correlated in some studies with a higher level of *HTRA1* mRNA and protein (Yang et al. 2006; Chan et al. 2007; Tuo et al. 2008). However, two recent studies did not confirm an association between the genotype at rs11200638 and *HTRA1* expression level in larger sample sets (Kanda et al. 2007; Chowers et al. 2008). These inconsistent results of polymorphism rs11200638 function reduce the plausibility for this variant in *HTRA1* as the AMD susceptibility factor. In *ARMS2*, the polymorphism rs10490924 (nonsynonymous A69S change) alone was reported to explain the bulk of the association between the 10q26 region and AMD (Kanda et al. 2007). In addition, *ARMS2* was also shown to locate to the mitochondrial outer membrane suggesting a mitochondrial pathway in AMD (Kanda et al. 2007). We recently demonstrated that strong LD in the region prevents the separation of an independent effect of rs10490924 from rs11200638 in our dataset (Wang et al. 2009). Furthermore, our results show that endogenous and exogenous *ARMS2* is not expressed in the mitochondria, but rather in the cytosol in an experimental system (Wang et al. 2009). Thus, based on the published genetic association and functional studies it is impossible to definitively determine the responsible gene at chromosome 10q26 locus.

Fritsche et al. recently reported a complex deletion/insertion polymorphism (443 bp deletion and in situ 54 bp insertion, indel) at the 3'UTR of *ARMS2* strongly associated with AMD. This indel reportedly dramatically decreases mRNA transcript level possibly by removing a proposed polyadenylation site and mediating rapid mRNA turnover (Fritsche et al. 2008). In an attempt to confirm and to extend the findings by Fritsche et al., we re-sequenced the indel in our sample set and attempted to replicate the downregulation of *ARMS2* transcription by the indel in both retina and blood samples.

Material and Methods

Samples

The case-control sample set contains 819 AMD cases and 329 unrelated controls for genotyping (Table 1). We recently started collecting PAXgene tube (Qiagen) from cases and controls for RNA extraction. We had only 46 cases and 6 unrelated controls with RNA extracted from peripheral blood for transcription analyzing. In the total 52 samples, 26 carry homozygous wild type alleles, 6 carry the homozygous indel alleles, and 20 samples were heterozygous. To sequence the *ARMS2* 3'UTR, we randomly choose 14 genomic DNA samples with genotype at polymorphism rs10490924 (4 GG, 5 GT and 5 TT). All participants are non-Hispanic Caucasians. All patients and controls received an eye examination and had fundus photographs graded according to a modified version of the age-related eye disease study (AREDS) grading system as described previously (Spencer et al. 2008). We include grades 1 and 2 as controls.

Grade 1 subjects have no evidence of drusen or small non-extensive drusen without pigmentary abnormalities, while grade 2 subjects 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 written informed consent, and this research adhered to the tenets of the Declaration of Helsinki.

Sequencing analysis

Genomic DNA was extracted from whole blood using the PureGene system (Gentra Systems) in the Biorepository Core in the John P. Hussman Institute for Human Genomics at University of Miami. Some of the DNA banking and genotyping was performed in the Center for Human Genetics Research DNA Resources Core at Vanderbilt University. DNA samples were sequenced at the Center for Genomic Technology of the John P. Hussman Institute for Human Genomics. Primers were designed according to the previously reported indel in the *ARMS2* 3' UTR and the flanking region (Fritsche et al. 2008). The PCR was applied by using the forward primer 5'-TGTCACCTGCATTCCCTCCTGTCAT-3' and the reverse primer 5'-AAGCTTCTTACCCTGACTTCCAGC-3'. The PCR products were separated in agarose gel and extracted, purified and cloned with TA cloning Kit (Invitrogen, Carlsbad, CA). The inserts were sequenced in both directions using the BigDye chemistry and an ABI3730 sequencer. Sequencing traces were analyzed using the Sequencer software (Ann Arbor, MI).

Taqman real-time PCR for indel genotyping

According to the sequence of the indel at *ARMS2* 3'UTR, two pairs of primers and probes were designed by Custom Taqman Copy Number Assays (Applied Biosystems). Primer pair A (Forward: CCTGCTGTAAAGGAGGTTACGA; Reverse: AGATGACTTCTCAGCTGGTTTCAC) targets the deleted sequence, while primer pair B (Forward: TGTTTTCTTGCCCTCCTTTCTCT; Reverse: CCTCAAGCCAGGTTGTCCTCTAGATCCAG) targets the two side flanking regions of the indel. The fluorescence generated during the PCR amplification was detected using the ABI Prism 7900HT sequence detection system and was analyzed with SDS2.3 and CopyCallerV1.0 software (Applied Biosystems). RNase P was amplified simultaneously as internal control to normalize the DNA input.

We verified that the A69S variant and the indel were in Hardy-Weinberg equilibrium (HWE) in controls, and we examined the linkage disequilibrium separately in cases and controls using Haploview software (data not shown). Using 819 AMD cases and 329 unrelated controls, we assessed the association of each variant and AMD risk using a chi-square test for allelic association.

RNA extraction from peripheral blood

RNA was extracted from whole bloods using the PAXgene Blood RNA System Kit (PreAnalytiX) according to the manufacturer's instructions. Briefly, 55 PAXgene tubes were removed from the -80°C freezer and incubated at room temperature overnight to ensure complete lysis. Following lysis the tubes were centrifuged at 5,000g for 10 min. 4 mL RNase-free water was added to the pellet, followed by vortexing and centrifugation at 5,000g for 10 min. The pellet was re-suspended in 350 µL of buffer BR1, and then 300 µL of BR2 lysis buffer

and 40 μ L of proteinase K were added. After digestion at 55 °C for 10 min, samples flowed through Shredder spin columns. The supernatant was added to 350 μ L ethanol and applied to RNA spin columns. After DNase digestion, column was washed and total RNA was eluted. Retinas were dissected from 24 human eyeballs and RNA was extracted by RNeasy lipid tissue kit (Qiagen) according to the manufacturer's instruction. The RNA kits (Agilent) for the 2100 bioanalyzer were used to monitor the quality and quantity of RNA. From 55 total samples, 52 samples successfully and clearly displayed a marker peak and two ribosome peaks (18S and 28S), and were utilized for further analysis.

RT-PCR for evaluating *ARMS2* expression across tissues

The retina RNA was purchased from Clontech. The blood RNA was in-house extracted from Paxgene blood tube. All other RNA samples were purchased from Ambion. The SuperScript III First-Strand Synthesis System (Invitrogen) was used for reverse transcription (RT) according to the manufacturer's instruction. Briefly, each RT reaction consisted of 200ng of purified RNA, random hexamer or Oligo(dT)20 Primers, dNTPs, 1 $\times$ RT buffer, MgCl₂ (5mM), DTT (50mM), SuperScript reverse transcriptase, and RNase'sOUT. RT reactions were incubated at 25°C for 10min, 50 °C for 50 min, 85 °C for 5 min, followed by adding 1 μ L RNase H and incubation at 37°C for 20 min. The PCR was applied by the following 3 pairs of primers,

ARMS2-forward: 5'-ATGCTGCGCCTATACCCAGG-3'

ARMS2-reverse: 5'-TTGCTGCAGTGTGGATGATAG-3'

β -actin-forward: 5'-GGGAAATCGTGCGTGACATTAAG-3'

β -actin-reverse: 5'-GTGTTGGCGTACAGGTCTTTG-3'

The PCR products were separated in 2% agarose gel. The gel image was captured by AlphaImager gel documentation system (San Leandro, CA). To further validate the specificity of *ARMS2* PCR, we have conducted a serial of control experiments: (1) after RNase digestion, we performed reverse transcription and PCR of *ARMS2*; (2) we directly PCR of *ARMS2* on RNA samples; (3) PCR with water.

Quantitative PCR for evaluating *ARMS2* expression in blood samples

Quantitative PCR was performed using an ABI HP7900. Within the plate, 52 samples were plated in duplicate in adjacent wells within the column; one for amplification of *ARMS2*, the other one for the housekeeper gene GAPDH as the internal control. Each sample was repeated three times at different locations in the 384-well plate. The sequences of pre-designed primers and probes for *ARMS2* and GAPDH (Applied Biosystems) are as follows,

ARMS2-forward: 5'-CCTGGAGTTGGTGGAGAAGGA-3'

ARMS2-reverse: 5'-GCTGGGATCATGGAGTGTGA-3'

ARMS2 probe: 5'-FAM-TTGCTCCTCTGCTTGTCAC-3'

GAPDH-Forward: 5'-GCACCACCAACTGCTTAGCA-3'

GAPDH-Reverse: 5'-GTCCTTCTGGGTGGCAGTGATG-3'

GAPDH-Probe: 5'-Vic-TCGTGGAAGGACTCATGACCACAGTCC-3'

The PCR reaction mix contains 20 $\times$ TaqMan Gene Expression Assay (1 μ L), 2 $\times$ master mix (10 μ L), cDNA (1 μ L corresponding to the cDNA reverse transcribed from approximately 10 ng RNA) and nuclease free water (8 μ L). The 384-well plate is then run on the 7900 HT at 50°C for 2 min, 95°C for 10 min, then 95°C for 15 s and 60°C for 1 min (for 40 cycles). After the PCR run is complete, quantitative gene expression data will be acquired and analyzed using the ABI Prism 7900HT Sequence Detection System (RQ manager). The experiment was

repeated twice. The *t* test was applied to compare the difference in the *ARMS2* level between the genotypes at the indel.

Results

Two side-by-side indels in *ARMS2* 3'UTR

To validate the indel, we sequenced *ARMS2* 3'UTR of 14 samples. We designed the PCR primers according to previously reported indel in the *ARMS2* 3'UTR and the flanking region (Fritsche et al. 2008). The expected PCR products are 209 bp for the indel allele and 598 bp for the wild type allele. The genotypes at SNP rs10490924 of the 14 samples for PCR are: 4 GG, 5 GT and 5 TT. By gel assay, all 5 TT samples show one band at ~200bp and all 4 GG samples show one band at ~600 bp. Out of the 5 samples that are heterozygous at rs10490924, 2 samples show only one band at ~200bp and 3 samples show 2 bands at ~200 and ~600bp (Fig 1A).

We then sequenced the bands of ~200 bp and ~600 bp. We found that the ~600 bp band is the wild type alleles compared to the genome database. The ~200 bp band indeed contains a 443bp deletion, 54bp insertion as reported (Fritsche et al. 2008). However, when aligning the indel sequencing data with the database, we found that there are actually two side-by-side indels in the 3'UTR of *ARMS2*: (1) 9 bp deletion at position of chr 10: 124206811~124206819 with adjacent 10 bp insertion; (2) 417 bp deletion at position of chr 10: 124206836~124207253 with adjacent 27 bp insertion. These two side-by-side indels are separated by a 17 bp (chr 10: 124206820~124206836) fragment (Fig 1B, C). From the sequence data of these 14 samples, we did not find any samples carrying only one side of the indel. All 8 indel samples sequenced contain both side-by-side indels.

The indel is associated with risk of AMD

To test whether the indel is associated with AMD, we genotyped a sample set of 819 cases and 329 unrelated controls. The indel was strongly associated with AMD (Table 2) confirming results reported by Fritsche et al. (Fritsche et al. 2008). However, the strong linkage disequilibrium (LD) between SNP rs10490924 and the indel ($D'=0.99$, $r^2=0.96$) in this sample makes it impossible to determine which variant is the biological relevant one by statistical modeling.

ARMS2 is ubiquitously expressed

ARMS2 is reported to be expressed only in human placenta and retina (Jakobsdottir et al. 2005; Fritsche et al. 2008). To investigate whether the 3'UTR indel decreases *ARMS2* mRNA stability, we screened *ARMS2* expression in 18 tissues using RT-PCR including different brain regions and blood. Unexpectedly, we found a specific band that showed in all of the 18 tissue samples (Fig 2A). We cloned and sequenced the bands. The sequence of the PCR band identically matched the *ARMS2* coding region (data not shown). Thus, *ARMS2* is ubiquitously expressed in human tissues including retina. After RNase digestion, RT-PCR of *ARMS2* showed no band. No bands showed in PCR with water either. And only weak bands showed in PCR directly with RNA samples (Fig 2B). These control experiments further suggest the specificity of *ARMS2* expression in human tissues.

The indel is not associated with lower level of *ARMS2* mRNA

To test the association between the genotypes at the indel with *ARMS2* mRNA level, we used RNA extracted from 24 control human retinas. The genotype at the indel was obtained by applying the PCR described above. Quantitative PCR show no significant difference ($P>0.05$) in the *ARMS2* mRNA level between 17 homozygous wild type (W) allele samples and 7

heterozygous (W-Id) samples (Fig 3A). To compensate the lack of homozygous Indel (Id) retinal samples, we applied quantitative PCR on RNA samples extracted from the peripheral white blood cells (WBC) which express relatively higher levels of *ARMS2* (Fig 2b). RNA was extracted from 52 samples (46 cases-6 controls). The dataset includes 26 homozygous wild type (W) alleles, 6 homozygous Indel (Id) alleles and 20 heterozygous samples. No significant difference ($P>0.05$) in the *ARMS2* mRNA level was found between the samples of different genotype at the indel of *ARMS2* 3'UTR (Fig 3B). No significant difference ($P>0.05$) of the *ARMS2* mRNA level was found between AMD affected and controls either (Fig 3C).

Discussion

Finding functional variants in the chromosome 10q26 region is one key to identifying which gene in the region confers risk for AMD. The rs11200638 risk allele in the *HTRA1* promoter was shown to associate with an increased expression by in vitro luciferase assay and immunostaining of human retinas (Yang et al. 2006; Chan et al. 2007; Tuo et al. 2008). However, this function of rs11200638 was not validated by a similar luciferase assay and quantitative PCR of blood samples in recent reports (Kanda et al. 2007; Chowers et al. 2008). Thus, it is still unclear whether rs11200638 is biologically involved in AMD.

Many nonsynonymous variants are involved in diseases by changing protein structure and functions. Exonic polymorphism rs10490924 generates an A69S change in *ARMS2*, making it a better candidate than *HTRA1*. Although we showed the exogenous *ARMS2* A69S variant is more likely to associate with the cytoskeleton than wild type, its biological function still remains to be elucidated.

The polymorphic indel at the *ARMS2* 3'UTR was associated with AMD in Caucasian and Japanese data sets (Fritsche et al. 2008; Gotoh et al. 2009). The recombinant deletion/insertion consists of a 443 bp deletion and an adjacent 54 bp insertion. We found that this indel is even more complex and is composed of two side-by-side indels separated by 17 bp. We have not found any samples so far that carry only one indel. It is unclear whether these two indels are completely linked or can be separated. The indel is strongly associated with AMD as reported (Fritsche et al. 2008). However, the strong linkage disequilibrium between these SNP rs10490924 and the indel makes it impossible to determine which variant is the biological relevant one by statistical modeling. Functionally, the indel (across the boundary of the 3'UTR tail and the flanking region) was thought to delete a polyadenylation site and insert two AUUUA motifs that are known to destabilize mRNA (Fritsche et al. 2008). The indel was subsequently associated with lower *ARMS2* transcript level by Northern blot of exogenous *ARMS2* mRNA from plasmid DNA transfection and Western blot of placenta samples. Neither of these two experiments directly measured the *ARMS2* mRNA level from retinal tissues.

A comprehensive characterization of the tissue distribution of *ARMS2* gene expression, unexpectedly revealed ubiquitous expression in human tissues including peripheral blood. By measuring *ARMS2* mRNA in the blood sample, we did not find any significant difference in the *ARMS2* mRNA level among the genotypes at the indel of *ARMS2* 3'UTR. Thus, contrary to a previous report (Fritsche et al. 2008), our results do not indicate that the indel causes unstable *ARMS2* transcripts in retina and blood samples. It remains unclear why deletion of a polyadenylation site and insertion of two AUUUA motifs do not significantly interfere with *ARMS2* transcripts in the white blood cells. We suggest, due to the strong linkage disequilibrium between the A69S variant and the indel, that it could be the A69S variant (Fritsche et al. 2008), not the indel, which confers risk of AMD.

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Figure 1.

Sequencing the indel at *ARMS2* 3'UTR. A, PCR of *ARMS2* 3'UTR. The upper band is the wild type allele and the lower band is the indel allele. All samples are labeled with their genotypes at SNP rs10490924. B, alignment of the indel sequence with the human genome. The red lower case letters are deleted nucleotides. The blue upper case letters are inserted nucleotides. The numbers at the left are the positions in the chromosome 10. C, Two side-by-side indels in the 3'UTR of *ARMS2* gene are separated by 17 bp.

Figure 2. *ARMS2* is ubiquitously expressed in human tissues. A, RT-PCR of *ARMS2* and β -actin in a list of tissue samples. B, No bands showed in PCR with RNase digested samples and water. Only weak bands showed in PCR with RNA.

Figure 3.

Quantitative PCR of *ARMS2* in human retina and blood samples. A, No significant difference of *ARMS2* mRNA level was found between 17 homozygous wild type (W) allele samples and 7 heterozygous human retinal samples ($P>0.05$). B, No significant difference of *ARMS2* mRNA level was found among the different genotypes at the 3'UTR indel of *ARMS2* in the white blood cells ($P>0.05$). C, No significant difference of *ARMS2* mRNA level in the white blood cells between AMD affected and controls ($P>0.05$).

Table 1

Characteristics of the study populations

Variable	Cases	Controls
Grade		
1		270
2		59
3	207	
4	114	
5	498	
total	819	329
Age of Exam (AOE $\pm$ sd)	76.1 $\pm$ 7.9	69.5 $\pm$ 9
Race, % Caucasian	100	100
Gender (% Female)	65.3	59

Table 2

Association of the indel in *ARMS2* 3'UTR and rs10490924 with AMD

Marker	Case MAF*	Control MAF	p-value	Age-adjusted Odds Ratio	95% Confidence Interval
rs10490924	0.412	0.248	1.89×10^{-13}	2.13	1.74 2.61
indel	0.409	0.248	3.62×10^{-13}	2.10	1.72 2.57

* MAF=minor allele frequency