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Variants at chromosome 10q26 locus and the expression of *HTRA1* in the retina

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Abstract

Variations in a locus at chromosome 10q26 are strongly associated with the risk of age-related macular degeneration (AMD). The most significantly associated haplotype includes a nonsynonymous SNP rs10490924 in the exon 1 of *ARMS2* and rs11200638 in the promoter region of *HTRA1*. It is under debate which gene(s), *ARMS2*, *HTRA1* or some other genes are functionally responsible for the genetic association. To verify whether the associated variants correlate with a higher *HTRA1* expression level as previously reported, *HTRA1* mRNA and protein were measured in a larger human retina-RPE-choroid samples (n = 82). Results show there is no significant change of *HTRA1* mRNA level among genotypes at rs11200638, rs10490924 or an indel variant of *ARMS2*. Furthermore, two AMD-associated synonymous SNPs rs1049331 and rs2293870 in *HTRA1* exon 1 do not change its protein level either. These results suggest that the AMD-associated variants in the chromosome 10q26 locus do not significantly affect the expression of *HTRA1*.

Identifying and verifying functional consequences of disease-associated genetic variants remains a challenge in the post-genome wide association study (GWAS) era. Two loci at chromosome 10q26 and 1q31 have been strongly associated with the risk of developing age-related macular degeneration (AMD), the principal cause for irreversible visual loss in developed countries. In contrast to the successful identification of complement factor H

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(*CFH*) at chromosome 1q31 as the first major susceptibility gene for AMD, there is a debate in the scientific community about functional variants and susceptibility genes in the chromosome 10q26 locus. Three genes are located within the bounds of the chromosome 10q26 locus, pleckstrin homology domain containing family A member 1 (*PLEKHA1*), *age-related maculopathy susceptibility 2 (ARMS2*, formerly *LOC387715*), and *HtrA serine peptidase 1 (HTRA1*), which are all associated with AMD. The most significantly associated haplotype includes single nucleotide polymorphisms (SNPs) rs10490924 (nonsynonymous substitution A69S) in *ARMS2* and rs11200638 in the promoter region of *HTRA1*. Some studies propose *ARMS2* as the AMD susceptibility gene, whereas others propose *HTRA1*. One major reason for the inconclusive findings is that variants in genes *ARMS2* and *HTRA1* are in such strong linkage disequilibrium (LD) that their effects are indistinguishable using statistical analysis.

HTRA1 was proposed as the susceptibility gene primarily based on the assumption that the expression of *HTRA1* is up-regulated by AMD-associated genotypes/haplotypes at the locus. SNP rs11200638 is located within a conserved CpG island and 497bp upstream from the transcription start site of *HTRA1*. Compared to the major allele G of rs11200638, the AMD-associated minor allele A may disrupt the CG pattern in the region, which is suggested to alter the transcription of *HTRA1*. One initial study showed that the risk allele of rs11200638 was correlated with a higher level of *HTRA1* mRNA in blood lymphocytes by quantitative RT-PCR and a higher level of *HTRA1* protein in human retinal pigment epithelium (RPE) by immunoblot (Yang et al., 2006). Subsequently, this correlation was replicated in archived retinas as well as fresh placenta tissues by immunohistochemistry and quantitative RT-PCR (Chan et al., 2007; Tuo et al., 2008; Yang et al., 2010). In contrast, several studies from independent groups, applying *ex vivo* and *in vitro* methods, have shown that the genotypes at rs11200638 or other AMD-associated variants in the chromosome 10q26 locus are not correlated with *HTRA1* expression at either mRNA or protein level in human retinas or other tissues (Kanda et al., 2007; Chowers et al., 2008; Wang et al., 2010a; Kanda et al., 2010; Friedrich et al., 2011). The discordant results call into question the proposed *HTRA1*-AMD functional association and call for more experiments to clarify whether variants at the chromosome 10q26 locus affect the expression of *HTRA1*.

One major reason for the discordant results is a lack of simultaneous quantification of *HTRA1* mRNA and protein in fresh human retinal tissues. To determine the relationship between variants at the chromosome 10q26 locus and *HTRA1* expression, we examined *HTRA1* in a larger sample of retina-RPE-choroids. A total of 82 human retina-RPE-choroids from 82 unrelated Caucasian subjects (age 75.0 ± 14.6 years old) without any known eye diseases were provided by the Florida Lions Eye Bank. Procedures for recruitment, requests for medical records, and consent forms were approved by the University of Miami, Miller School of Medicine Institutional Review Board. Eye tissues were retrieved and frozen in −80°C within 24 hrs post-mortem. Retinal tissues (including neuroretina, RPE and choroid) were punched from the macular region of frozen eyes for RNA and protein extractions. RNA was extracted by RNeasy lipid tissue kit (Qiagen) and protein was extracted by RIPA buffer (Thermo) according to the manufacturer's instructions. The quality and quantity of RNA were monitored by a Bioanalyzer 2100 (Agilent) and a NanoDrop 8000

Spectrophotometer (Thermo). The concentration of total protein was measured using a BCA protein assay kit (Thermo).

Three variants, including rs10490924, rs11200638 and *ARMS2* 3′UTR indel (a combination polymorphism of insertion and deletion) at the chromosome 10q26 locus have previously been strongly associated with AMD. All samples were genotyped for these variants. One other SNP rs2736911 (R38X in *ARMS2*) was reported to be associated inversely with AMD (Yang et al., 2010). We recently showed that this inverse association is insignificant after adjustment for sex and age. Additional analyses further suggested that the trending inverse association of rs2736911 (without adjustment for sex and age) appears to be due to strong LD with the non-risk wild-type allele at rs10490924 (Wang et al., 2012). For this reason, rs2736911 was not included in this study. Genotypes at SNPs rs10490924 and rs11200638 were assessed by Taqman assays (Life Technologies). Genotypes at the *ARMS2* 3′UTR indel were evaluated by PCR and gel assay as described previously (Fritsche et al., 2008; Wang et al., 2010b). The three variants are located in a strong LD region. Overall, samples include 54 individuals homozygous for protective haplotype (G-WT-G), 24 heterozygotes and 4 individuals homozygous for risk haplotype (T-indel-A).

To test whether the risk haplotype is associated with a higher expression of *HTRA1* as previous reported (Yang et al., 2010), primers (forward: 5′-CGGAAGATGGACTGATCGTGAC-3′; reverse: 5′-GGTGATGGCTTTTCCTTTGGC-3′) were applied for RT-PCR analysis. Twelve retina-RPE-choroid samples included 4 individuals homozygous for protective haplotype (G-WT-G), 4 heterozygotes, and 4 individuals homozygous for risk haplotype (T-indel-A). There was no significant difference in age among the three groups (data not shown). RT-PCR of housekeeper gene *GAPDH* was used for internal controls. PCR products were displayed at 2% agarose gel (Figure 1A) and the band density was quantified by AlphaImager software. After normalization with GAPDH band densities, no statistically significant difference was identified ($P > 0.05$) in *HTRA1* RT-PCR bands among haplotypes of the three chromosome 10q26 locus variants using Student *t* test (Figure 1B).

To more accurately quantify *HTRA1* mRNA level, we performed quantitative RT-PCR using a Taqman gene expression assay (Life Technologies) for all 82 retina-RPE-choroid samples. There was no significant difference in age among the three haplotype groups (data not shown). The quantitative RT-PCR reaction mix contains 20× TaqMan Gene Expression Assay (1μl), 2× master mix (10μl), cDNA (1μl corresponding to the cDNA reverse transcribed from approximately 25 ng RNA) and nuclease free water (8μl). Each sample was repeated three times at different locations in the plate. The 384-well plate was then run on the 7900 HT (Life Technologies) at 50°C for 2 min, 95°C for 10 min, then 95°C for 15 s and 60°C for 1 min (for 45 cycles). The *HTRA1* expression level was calculated using the $2^{-\Delta\Delta Ct}$ method and normalized by *GAPDH* as internal controls. We did not find statistically significant differences ($P > 0.05$) in *HTRA1* expression level among haplotypes of the three chromosome 10q26 locus variants using the Student *t* test (Figure 1C).

Previous studies examined *HTRA1* mRNA in smaller samples with only a few samples homozygous for the risk haplotype. From 10 retinas including one sample homozygous for

AA genotype rs11200638, Chan et al. observed a trending increase of *HTRA1* mRNA with risk genotype at rs11200638 (Chan et al., 2007). By examining 35 retinas including six samples (5 controls and 1 AMD affected) homozygous for risk haplotype, Kanda et al found no association between AMD susceptibility variants at chromosome 10q26 and the expression level of *HTRA1* (Kanda et al., 2010). Friedrich et al. quantified *HTRA1* mRNA in 45 human retinas including 2 samples homozygous for risk haplotype and stated that the risk haplotype does not affect *HTRA1* expression (Friedrich et al., 2011). Our group previously reported that there were no effects of rs11200638 genotype on *HTRA1* expression by examining 24 retina-RPE-choroids (no homozygous risk allele sample included) (Wang et al., 2010a). In this updated sample set of 82 retina-RPE-choroids including 4 samples homozygous for risk haplotype, we found no statistically significant correlation between AMD-associated haplotype at chromosome 10q26 and the *HTRA1* transcripts level. The result from these 4 groups suggests that variants at the chromosome 10q26 locus do not likely affect the transcription of *HTRA1* in the retina.

After evaluating the effect of upstream variants on *HTRA1* transcription, we turned our focus on the two synonymous SNPs rs1049331 (A34A) and rs2293870 (G36G) in exon 1 of the *HTRA1* gene. SNPs rs1049331 and rs2293870, located within a strong LD with rs11200638 and other variants, also associated with risk of AMD or AMD sub-phenotypes (Deangelis et al., 2008; Tam et al., 2008; Andreoli et al., 2009). Although one synonymous SNP will not result in an amino acid change, there is a possibility that the two synonymous SNPs may alter *HTRA1* translation by disruptions in *HTRA1* mRNA structure or tRNA preferences as previously reported in other genes (Lavner and Kotlar 2005; Chamary et al., 2006; Sauna et al., 2011). To explore this possibility, we first conducted *in silico* analysis. We found that SNPs rs1049331 and rs2293870 cause slight changes in the secondary structure of *HTRA1* mRNA predicted by a CentroidFold algorithm (Hamada et al., 2009) (Figure 2A, B). We then applied immunoblot to semi-quantify HTRA1 protein in retina-RPE-choroids. Another set of punches was collected for protein extraction from the same 12 eye tissues used for RT-PCR analysis (Figure 1A). Genotypes at rs1049331 and rs2293870 were obtained by PCR using primers (forward: 5′-AGAGTCGCCATGCAGATCC-3′; reverse: 5′-CACAGGTTGGCGTAGGTGTT-3′) and sequencing. HTRA1 antibodies (monoclonal from R&D or polyclonal from Abcam) were used in this experiment. HTRA1 band density was quantified by AlphaImager software and normalized normalization by β-actin band densities (Figure 2C). No statistically significant difference was identified ($P > 0.05$) in *HTRA1* protein bands among genotypes at rs1049331 and rs2293870 (Figure 2D). Risk genotype AA at rs11200638 or the aforementioned risk haplotype is not associated with a higher HTRA1 protein level in the retina-RPE-choroid either.

A 1.7-fold increase of HTRA1 protein level was initially reported in wet AMD affected RPE samples with genotype AA (n = 4) compared to control RPE samples of genotype GG (n = 6) at rs11200638 by immunoblot analysis (Yang et al., 2006). However, the correlation between genotypes at rs11200638 and HTRA1 protein level is not verified in our experiments. By coordinating *HTRA1* mRNA and protein level, our results further suggest that variants at chromosome 10q26, including the two synonymous SNPs, may not affect

HTRA1 protein level. Further studies are needed to examine the relationship between variants at chromosome 10q26 and *HTRA1* at the protein level in the retina.

A recent systemic survey for biomarkers of AMD revealed many genes that are either over- or under-expressed in AMD-affected RPE/choroid. *HTRA1* was not on the list of those differentially expressed genes (Newman et al., 2012). Interestingly, overexpressing *HTRA1* specifically in mouse RPE induces some phenotypes relevant to AMD in humans (Vierkotten et al., 2011; Jones et al., 2011). However, if chromosome 10q26 locus variants do not change the transcription, translation or other functions of *HTRA1*, it could be other genes at this locus underlying the susceptibility to AMD.

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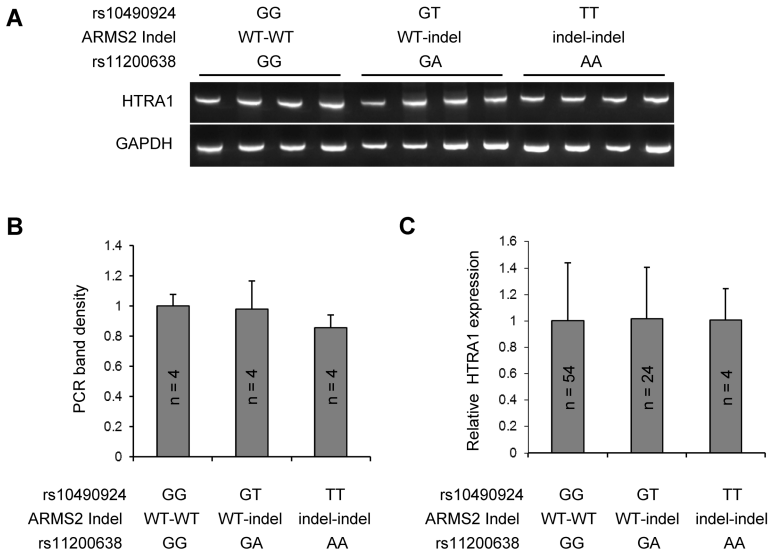

Figure 1.

Variants at the chromosome 10q26 locus and the level of *HTRA1* mRNA. A. Agarose gel images of the RT-PCR of *HTRA1* and GAPDH in 12 human retina samples including 4 individuals homozygous for protective haplotype (G-WT-G), 4 heterozygotes and 4 individuals homozygous for risk haplotype (T-indel-A). B. Quantification of RT-PCR band densities. No statistically significant difference was found ($P > 0.05$) in *HTRA1* RT-PCR bands among haplotypes of the three chromosome 10q26 locus variants. C. Analysis of quantitative RT-PCR of *HTRA1* and GAPDH in 82 human retina samples including 54 individuals homozygous for protective haplotype, 24 heterozygotes and 4 individuals homozygous for risk haplotype. *HTRA1* mRNA levels are not significantly different ($P > 0.05$) among haplotypes of the three chromosome 10q26 locus variants.

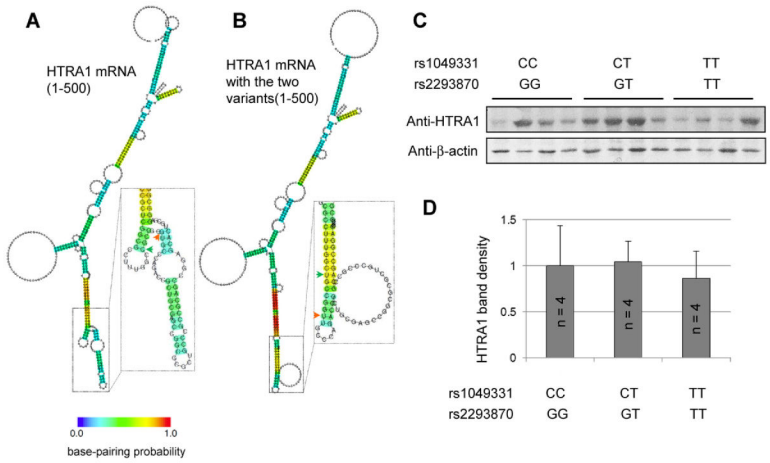

Figure 2.

Variants at the chromosome 10q26 locus and the level of *HTRA1* protein. A. Secondary structure of *HTRA1* mRNA (1–500 nt) predicted by CentroidFold. B. Secondary structure of *HTRA1* mRNA (1–500 nt) carrying minor alleles at two synonymous SNPs rs1049331 (green arrow head) and rs2293870 (orange arrow head). There are slight structural changes caused by the two SNPs showing in the boxes. C. Immunoblot analysis of *HTRA1* and β-actin. D. Quantification of immunoblot band densities. There is no statistical analysis among genotypes at the chromosome 10q26 locus variants.