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Htra1 Expression In Humanized Arms2 Knock-In Mouse Models

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Htra1 expression in humanized *ARMS2* knock-in mouse models

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Abstract

Age-related macular degeneration (AMD) is the most common cause of central vision loss in developed world. Two genes on chromosome 10q26, maculopathy susceptibility 2 (*ARMS2*) and HtrA serine peptidase 1 (*HTRA1*) were identified as candidate genetic factors of AMD. However, due to the high linkage disequilibrium across the locus as well as the inconsistent functional findings regarding the two genes, it is difficult to distinguish the causative gene that confer the risk of AMD. To provide insight of the functional roles of *ARMS2* and *HTRA1* in the pathogenesis of AMD, we investigated the regulation relationship between the two genes in vivo. To overcome the difficulty that *ARMS2* gene only exists in higher primates, we generated humanized *ARMS2* knock-in mice. Human *ARMS2* cDNA was inserted into the corresponding locus upstream the *Htra1* gene in mouse genome. Decline of *Htra1* gene expression was found in the cortex of *ARMS2* KI mice using RT-qPCR technique. Our finding indicates the presence of *ARMS2* gene upstream *Htra1* may manifest negative regulation effect upon *Htra1* expression. This new evidence revealed the complexity of how the two genes might work together in the causal pathway of AMD. Furthermore, we provided a novel and valuable animal model that could facilitate further research of AMD pathogenesis.

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Introduction

Age-related macular degeneration (AMD) is a chronic disease which accounts for 8.7% of all blindness worldwide and the most prevalent cause of blindness in developed countries especially among the elders [1][2][3]. AMD affects the central region of retina (macula) which is responsible for visual acuity and color vision. As a chronic disease, AMD can be classified into early, intermediate and advanced stage. Advanced AMD can be further divided into two subtypes: geographic atrophy (late-stage dry AMD) and neovascular AMD (wet AMD). Late-stage dry AMD results in loss of retinal pigment epithelium (RPE) cell and overlying photoreceptors which leads to a progressive visual impairment [4]. For wet AMD, new vessels sprout from choroid and break beneath the retina, which causes permanent damage to adjacent retinal tissue and leads to central vision loss [5].

The pathogenesis of AMD is poorly understood. It likely results from the combination of multiple environmental and genetic factors. Notable environmental risk factors include smoking, diet, alcohol use and infections [6]. Several AMD susceptibility loci were also identified through genetic studies. Two chromosome regions, 1q31 and 10q26, were repeatedly associated with AMD in most studies and confirmed by a meta-analysis [7]. In 2005, several studies reported complement factor H (*CFH*) gene located on chromosome 1q31 as the first AMD susceptibility gene and *CFH* Y402H was recognized as a major AMD susceptibility variant [8][9]. On the other hand, several AMD risk variants were found in a high linkage disequilibrium (LD) region on chromosome 10q26. This region encompasses three genes: pleckstrin homology domain-containing A1 (*PLEKHA1*), age-related maculopathy susceptibility 2 (*ARMS2*, formerly named

LOC387715), and HtrA serine peptidase 1 (*HTRA1*), which make it difficult to identify which gene(s) is the causal one(s) [10]. Jakobosdottir et al. (2005) suggested *PLEKHA1* is the major contributor to AMD susceptibility [11]. Rivera et al. (2005) found the strongest association was centered over a coding SNP rs10490924 within *ARMS2* [12]. Later, DeWan et al. (2006) and Yang et al. (2006) identified rs11200638 in the *HTRA1* promoter as the most significantly associated variant of AMD and suggested that increasing *HTRA1* expression may play a role in AMD pathogenesis [5][13]. However, Kanda et al. (2007) showed that rs10490924 in *ARMS2* alone can explain the AMD susceptibility, contradicted with former findings [14]. Since then, evidence have emerged to demonstrate the strong association of rs10490924 and rs11200638 with the risk of AMD in different studies, respectively. A meta-analysis summarized the results and confirmed the association with AMD risk for both rs10490924 and rs11200638, suggesting a moderate codominant, multiplicative genetic mode of *ARMS2* and *HTRA1* in the AMD pathogenesis [15]. Besides, another two important AMD susceptibility variants have been identified: the indel polymorphism in the 3' -UTR of *ARMS2* and the nonsense polymorphism rs2736911 (C>T: R38X) in the first exon of *ARMS2* [16].

Because of the high LD across the *ARMS2-HTRA1* locus, statistical genetics method alone cannot determine which variant(s) is causative for AMD risk. Therefore, molecular functional studies are required to investigate their roles in AMD pathogenesis. Here we review the existing knowledge of *ARMS2* and *HTRA1* about their molecular function with regard to AMD.

ARMS2

ARMS2 is a primate-specific gene encoding a 11-kDa protein ARMS2 [17]. It expresses strongly in the placenta but weakly in retinal tissues [18]. The structure, cellular localization and function of the protein are unclear because of contradictory results. A recent study suggested it may function in complement activation process; deficiency of this protein may reduce the clearance of cellular debris and enhance formation of drusen, the hallmark of AMD [19].

The effect of rs10490924 on the structure and function of *ARMS2* is unclear. Early evidence indicated that it may cause a nonsynonymous change A69S in ARMS2 protein [14]. The indel polymorphism in the 3' -UTR of *ARMS2* has been reported to destabilize *ARMS2* transcript and strongly associated with AMD [20]. The nonsense polymorphism rs2736911 (C>T: R38X) in the first exon of *ARMS2* was believed to introduce a premature stop codon and eventually lead to less ARMS2 protein. However, this variant is not positively associated with AMD, in contrast to the indel variant [18].

HTRA1

HTRA1 gene encodes a 51-kDa secreted serine protease (HTRA1) which is widely expressed in many types of tissue including retina. HTRA1 protein can degrade extracellular matrix (ECM) proteins as well as regulate signaling of transforming growth factor-beta (TGFβ) family protein, insulin-like growth factor (IGF) and fibroblast growth factors (FGF), playing important role in cell proliferation and migration [10][21][22]. Human *HTRA1* gene has been linked with the microvascular and macrovasculature diseases including arthritis, preeclampsia and tumor progression besides AMD [23].

There is a hypothesis about how HTRA1 may involve in the pathogenesis of AMD: overexpressed HTRA1 protein would cause excess digestion of ECM, leading to elevated levels of pro-inflammatory peptide fragments that increase oxidative stress and eventually contributing to AMD [16].

The AMD-associated variant rs11200638 in *HTRA1* promoter has been initially suggested to increase *HTRA1* expression [5][13]. Several follow up studies, on the contrary, showed that rs11200638 may not increase *HTRA1* expression level [14][24]. Overall, the results of how rs11200638 might affect *HTRA1* expression remains inconsistent. Besides, the adjacent indel polymorphism in the 3' -UTR of *ARMS2* was suggested to have joint effect with rs11200638 to regulate *HTRA1* expression [16][25][26].

The genetic studies and functional studies till now have not yet reached a consensus about the casual gene associated with AMD. Inspired by previous studies, especially for those implied *ARMS2* (and its downstream sequence) might regulate *HTRA1* expression, we think exploring the functional relationship between *ARMS2* and *HTRA1 in vivo* might shed light on solving the "causative" puzzle.

However, *ARMS2* is a gene only present in Old World monkeys and apes besides human [27], which make it difficult to investigate its function in commonly used animal model such as mice. Several groups have created *ARMS2* transgenic mice [28][29]. In those mice, human *ARMS2* gene was randomly inserted into their genome, which makes the interaction between *ARMS2* and *Htra1* cannot be well examined. Besides, these mice appeared normal and didn't develop any typical AMD-related cellular or molecular phenotype, indicating that proper genomic location of *ARMS2*

upstream *Htra1* promoter might be crucial for the hypothetical regulation effect. Therefore, we generated humanized *ARMS2* knock-in mice by inserting human *ARMS2* cDNA into the corresponding locus upstream the *Htra1* gene in mouse genome. By examining *Htra1* expression in humanized *ARMS2* knock-in mice, we could understand the regulation relationship between these two genes.

Materials and Methods

General study design

Our goal is to detect possible *Htra1* expression change in humanized *ARMS2* knock-in mice. In order to make meaningful comparisons, two genotypes of mice with humanized *ARMS2* sequence were constructed. Genotyping was performed to identify all mice's genetic makeup. RNA was extracted from cortex or hippocampus from subject mice. Cortex and hippocampus were chosen because previous results in our lab (*in situ* hybridization) showed that *Htra1* expression only presents in these two regions in mouse brain. RT-qPCR, a widely-used method of quantitating gene expression, was conducted to measure *Htra1* expression in transcriptional level among different groups. Data from RT-qPCR was collected and analyzed to compare the gene expression level.

Animals

Three genotypes of mice were generated with the help of Ozgene (Perth, Australia) for further experiment:

***ARMS2* flox mice (*ARMS2*$^{flox/flox}$)**

***ARMS2* knock-in (KI) mice (*ARMS2*$^{KI/KI}$)**

Wild type (WT) mice

Similar process was described in our previous study [30]. A targeting vector using mouse *Htra1* upstream homologous sequence was created (Figure 1). A Human ubiquitin C (UbiC) promoter, a transcriptional stop sequence flanked by loxP sites, a human *ARMS2* cDNA tagged with a flag epitope, and a flippase recognition target (FRT)-flanked phosphoglycerate kinase-1 (PGK)-neomycin cassette, were inserted upstream of *Htra1* promoter. The vector was then electroporated into embryonic stem (ES) cells from C57BL/6 mice to generate chimeric mice. Further breeding was done to create the three final genotypes. *ARMS2* flox allele was created by removing selection cassette through breeding with OzFlpE mice (*FlpE* knock-in mice). *ARMS2* KI allele was further generated by removing the loxP-flanked stop sequence through crossing to germline-transmitting Rosa26-Cre mice. The WT mice and *ARMS2* flox mice are littermate. All mice were kept in C57BL/6 genetic background. All animal experiments were conducted with the help of Dr. Jianyu Wu in accordance with Statement by Yale University on the Humane Use of Animals in Research and Education.

As a result, *ARMS2* should not be expressed in *ARMS2* flox mice because of the transcriptional stop sequence, while *ARMS2* can be ubiquitous constitutively expressed

in *ARMS2* KI mice. The rationale of this construction is to distinguish whether the possible *Htra1* expression level change derives from active *ARMS2* gene expression (transcriptional or post-transcriptional level) or genome structural change upstream *Htra1*.

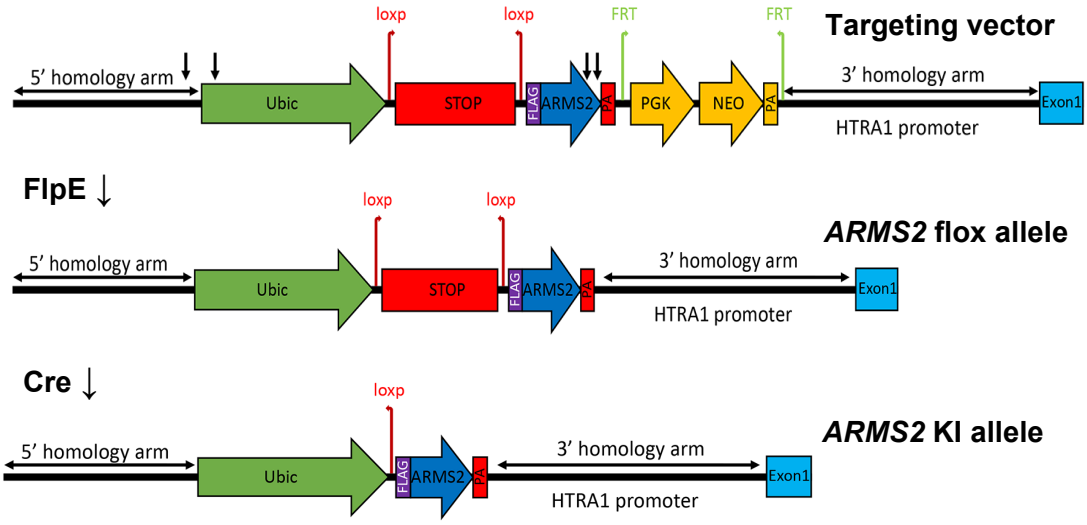

Figure 1 Construction of humanized *ARMS2* knock in mice

A Ubic promoter, a stop sequence flanked by loxP sites, a human *ARMS2* cDNA tagged with a flag epitope and a FRT flanked neo cassette was inserted 5' to *Htra1* promoter. Expression of *FlpE* removes the neomycin selection cassette. Expression of *Cre* causes excision of the stop sequence.

Genotyping

Polymerase Chain Reaction (PCR) was performed to genotype each mouse: REDExtract-N-Amp Tissue PCR Kit (Sigma) was used to extract DNA from mouse toe tissue and perform further PCR process. Specific primer combinations were designed to distinguish different alleles (Table 1). The expected bands for WT, *ARMS2* flox and *ARMS2* KI alleles are 200 bp, 340 bp and 470 bp, respectively. Conventional PCR program was used to amplify DNA (Table 2). Agarose gel (2%) electrophoresis was performed to distinguish amplified DNA bands.

Table 1 PCR primers for genotyping

Alleles	Primers
ARMS2 flox allele	5'-CTTTGGCAGCTATGGTGTCTCACCA-3'
	5'-ACTTCTCATCCATCAGGTCTTGGCA-3'
	5'-CCTGTTCCGCTCTCTGGAAAGAAAA-3'
ARMS2 KI allele	5'-CTTTGGCAGCTATGGTGTCTCACCA-3'
	5'-ACTTCTCATCCATCAGGTCTTGGCA-3'
	5'-TATCATCCACACTGCAGCAAGGTGA-3'

Table 2 PCR program for genotyping (conventional PCR)

Temperature	Time	Cycles
94 ˚C	5 minutes	
94 ˚C	30 seconds	35 cycles
60 ˚C	30 seconds	
70 ˚C	60 seconds	
70 ˚C	5 minutes	
16 ˚C	forever	

RNA extraction

All mice were sacrificed around 18 to 20 days after birth. Cortex and hippocampus tissues were dissected out from each mouse after perfusing them with Phosphate Buffered Saline (PBS). Tissues were then quickly frozen on dry ice and stored at -80°C, for preventing RNA degradation caused by RNase.

RNA was then extracted from those tissues. First, cortex or hippocampus tissues were separately homogenized with motor pestles and dissolved in TRIzol (ThermoFisher Scientific). Then, RNA was extracted by phenol-chloroform extraction and precipitated by isopropyl alcohol. The RNA pellet was resuspended in RNase-free water. RNA concentration was measured by Nanodrop spectrophotometer (Fisher Scientific).

RT-qPCR

Real-time quantitative polymerase chain reaction (RT-qPCR) is a definitive technique for quantitating differences in gene expression levels between samples [31]. The basic principle is as follow: first, fluorescent double-stranded (ds) DNA-binding dye SYBR® Green was added into PCR system. Then the reaction was run in a RT-qPCR instrument, in which the intensity of fluorescence after each cycle was measured. Because SYBR® Green only emits fluorescence after binding with dsDNA, amplification of templates can be quantitating through measuring the fluorescence. Usually, a threshold for detection of fluorescence will be set up. The cycle number at which the fluorescence reaches the threshold is called the Threshold Cycle (C_t). To measure gene expression, the C_t of a housekeeping gene (for example, *β-actin*) from the same sample

will be measure as well, in order to normalizing the variation of RNA amount among different samples. The relative expression level can be calculated as follow:

$$R = \frac{\mathrm{I}_{\text{interested gene}}}{\mathrm{I}_{\text{housekeeping gene}}} = \frac{\mathrm{T}/2^{\mathrm{Ct}_{\text{interested gene}}}}{\mathrm{T}/2^{\mathrm{Ct}_{\text{housekeeping gene}}}} = 2^{Ct_{\text{housekeeping gene}} - Ct_{\text{interested gene}}} = 2^{\Delta Ct}$$

(R=relative expression level; I=initial copy number; T=threshold; C_t=threshold cycle;)

In our study, RT-qPCR was used for quantitating the relative expression level of both *ARMS2* and *Htra1* gene. First, same amount of RNA extracted from tissues was used as template to reverse-transcribe to cDNA using iScriptTM Reverse Transcription Supermix (Bio Rad). *β-actin* was used as housekeeping gene to normalize the RNA expression level. Different primer combinations were designed for *Htra1*, *β-actin or ARMS2* gene (Table 3). RT-qPCR was performed using CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). SYBR® Green JumpStart™ (Sigma) was used as RT-qPCR fluorescent dye. RT-qPCR program is list in Table 4.

For each RNA sample, the reverse transcription process was duplicated. For each cDNA sample, RT-qPCR was carried out three times in parallel. C_t was measured for each run. The whole RT-qPCR experiment was thoroughly duplicated for valid result.

Table 3 RT-qPCR primers for *Htra1*, *β-actin* and *ARMS2*

Genes	Primers
Htra1	5'-TTGCTGATGTGGTGGAGAAG-3'
	5'-CAATCAGTCCATCCTCCGATAC-3'
β-actin	5'-GCCACCAGTTCGCCATGGA-3'
	5'-TACAGCCCGGGGAGCATCGT-3'
ARMS2	5'-GTAAATTGTCCGCTAAATTCTGGCC-3'
	5'-TTGTCGTCATCGTCCTTGTAGT-3'

Table 4 RT-qPCR program

Temperature	Time	Cycles
94 ˚C	2 minutes	
94 ˚C	15 seconds	40 cycles
60 ˚C	60 seconds	

Data analysis

As the flow diagram (Figure 2) show, after RNA extraction, each RNA sample generated two cDNA samples. Each cDNA sample underwent 3 parallel RT-qPCR, generating 3 relative expression data (R1-R3). Mean 1 or Mean 2 is the average of each triplicate RT-qPCR results. The whole experiment was duplicated, thus, two more average expression data, Mean 3 and Mean 4, were generated. The final data for each tissue sample from different mice we used to make comparisons, was the average of the Mean 1 to Mean 4.

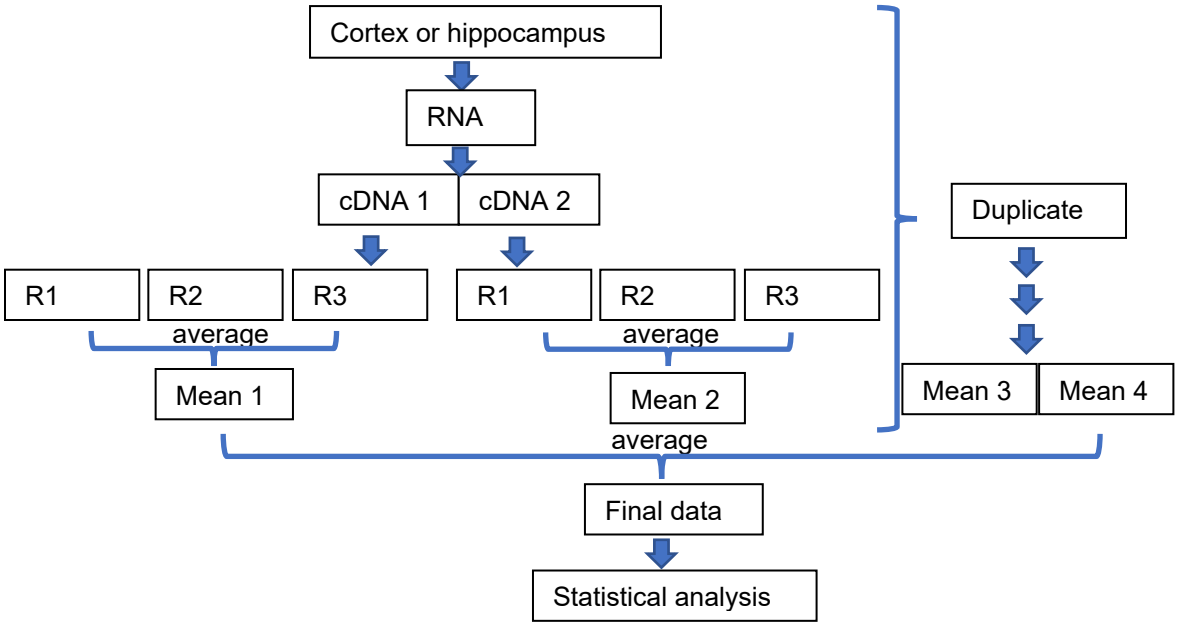

Figure 2 Diagram of RT-qPCR data generating

Analysis of variance (ANOVA) was performed using the final average relative expression level derived from *ARMS2* flox mice, *ARMS2* KI mice and wild type mice to detect the difference of *Htra1* expression. The Fisher Least Significant Difference (LSD) method was used for multiple comparisons. All statistical analysis was done using IBM® SPSS® Statistics 25 and GraphPad Prism 9.

Results

Genotyping

The genotyping result is listed in Table 5. All of them were early adult mice aged 18 to 20 days after birth. 7 wild type mice, 7 *ARMS2* flox mice and 9 *ARMS2* KI mice were used for further experiments.

Table 5 Mouse information and genotyping

Mouse number	Date of Birth	Sacrifice date	Postnatal Date	genotype
369	8/28/2019	9/18/2019	20	WT
370	8/28/2019	9/18/2019	20	WT
490	9/14/2019	10/2/2019	18	WT
494	9/14/2019	10/2/2019	18	WT
496	9/14/2019	10/2/2019	18	WT
498	9/12/2019	10/2/2019	20	WT
503	9/12/2019	10/2/2019	20	WT
372	8/28/2019	9/18/2019	20	*ARMS2* flox
373	8/28/2019	9/18/2019	20	*ARMS2* flox
487	9/14/2019	10/2/2019	18	*ARMS2* flox
489	9/14/2019	10/2/2019	18	*ARMS2* flox
491	9/14/2019	10/2/2019	18	*ARMS2* flox
497	9/12/2019	10/2/2019	20	*ARMS2* flox
502	9/12/2019	10/2/2019	20	*ARMS2* flox
249	2/24/2020	3/13/2020	18	*ARMS2* KI
251	2/24/2020	3/13/2020	18	*ARMS2* KI
253	2/24/2020	3/13/2020	18	*ARMS2* KI
254	2/24/2020	3/13/2020	18	*ARMS2* KI
255	2/24/2020	3/13/2020	18	*ARMS2* KI
726-1	7/7/2020	7/26/2020	19	*ARMS2* KI
726-2	7/7/2020	7/26/2020	19	*ARMS2* KI
726-3	7/7/2020	7/26/2020	19	*ARMS2* KI
726-4	7/7/2020	7/26/2020	19	*ARMS2* KI

Detection of *ARMS2* mRNA

ARMS2 mRNA was detected in cortex and hippocampus from *ARMS2* KI mice, while no signal was detected in cortex or hippocampus from *ARMS2* flox mice or WT mice (Table 6). The result showed *ARMS2* expresses in cortex and hippocampus of *ARMS2* KI mice but not in other mice. It confirmed that our humanized *ARMS2* KI mouse model was well constructed. On the other hand, *ARMS2* flox mice, despite harboring *ARMS2* cDNA, doesn't actively express the gene.

Table 6 *ARMS2* mRNA relative expression in cortex and hippocampus (final data)

Mouse number	Genotype	*ARMS2* Expression in Cortex	*ARMS2* Expression in Hippocampus
490	WT	Not detectable	Not detectable
494	WT	Not detectable	Not detectable
496	WT	Not detectable	Not detectable
498	WT	Not detectable	Not detectable
503	WT	Not detectable	Not detectable
373	*ARMS2* flox	Not detectable	Not detectable
487	*ARMS2* flox	Not detectable	Not detectable
489	*ARMS2* flox	Not detectable	Not detectable
497	*ARMS2* flox	Not detectable	Not detectable
502	*ARMS2* flox	Not detectable	Not detectable
255	*ARMS2* KI	.00048379	.00025691
726-1	*ARMS2* KI	.00022148	.00032346
726-2	*ARMS2* KI	.00031695	.00051378
726-3	*ARMS2* KI	.00038163	.00042213
726-4	*ARMS2* KI	.00030829	.00046725

Quantification of *Htra1* expression

Cortex

Htra1 relative expression level among different mice in cortex was shown in Table 7 and Figure 3. Test of homogeneity of variance showed an insignificant result (p=0.411), thus, one-way ANOVA is proper to use for comparison. ANOVA showed that there was a significant difference (p=0.029) of *Htra1* relative expression level among the three groups of mice. Multiple comparisons revealed that the difference between *ARMS2* KI mice and WT mice (p=0.011), and the difference between *ARMS2* flox mice and WT mice (p=0.041) are both significant. Comparing to WT mice, *Htra1* expression in *ARMS2* KI mice and *ARMS2* flox mice decreased by 22% and 18% in average, respectively. There was no significant difference between *ARMS2* KI mice and *ARMS2* flox mice regarding *Htra1* relative expression level (p=0.641).

Hippocampus

Htra1 relative expression level among different mice in hippocampus was shown in Table 7 and Figure 4. Test of homogeneity of variance showed an insignificant result (p=0.529), thus, one-way ANOVA is proper to use. However, ANOVA detected no significant difference of *Htra1* relative expression level among the three groups of mice (p=0.143).

Table 7 *Htra1* mRNA relative expression in cortex and hippocampus (final data)

Mouse number	Genotype	*Htra1* Expression in Cortex	*Htra1* Expression in Hippocampus
369	WT	.10725	.11075
370	WT	.12035	.11759
490	WT	.11414	.10032
496	WT	.11315	.13843
498	WT	.11933	.12115
494	WT	.07882	.06100
503	WT	.13300	.13683
372	*ARMS2* flox	.11401	.09589
373	*ARMS2* flox	.08579	.09036
487	*ARMS2* flox	.08761	.08615
489	*ARMS2* flox	.12138	.11443
497	*ARMS2* flox	.07164	.07949
491	*ARMS2* flox	.06083	.05842
502	*ARMS2* flox	.10103	.07959
249	*ARMS2* KI	.10579	.10705
251	*ARMS2* KI	.08870	.11277
253	*ARMS2* KI	.07534	.08932
254	*ARMS2* KI	.08471	.15546
255	*ARMS2* KI	.11319	.07150
726-1	*ARMS2* KI	.07224	.08752
726-2	*ARMS2* KI	.09362	.12657
726-3	*ARMS2* KI	.07495	.08099
726-4	*ARMS2* KI	.07938	.09463

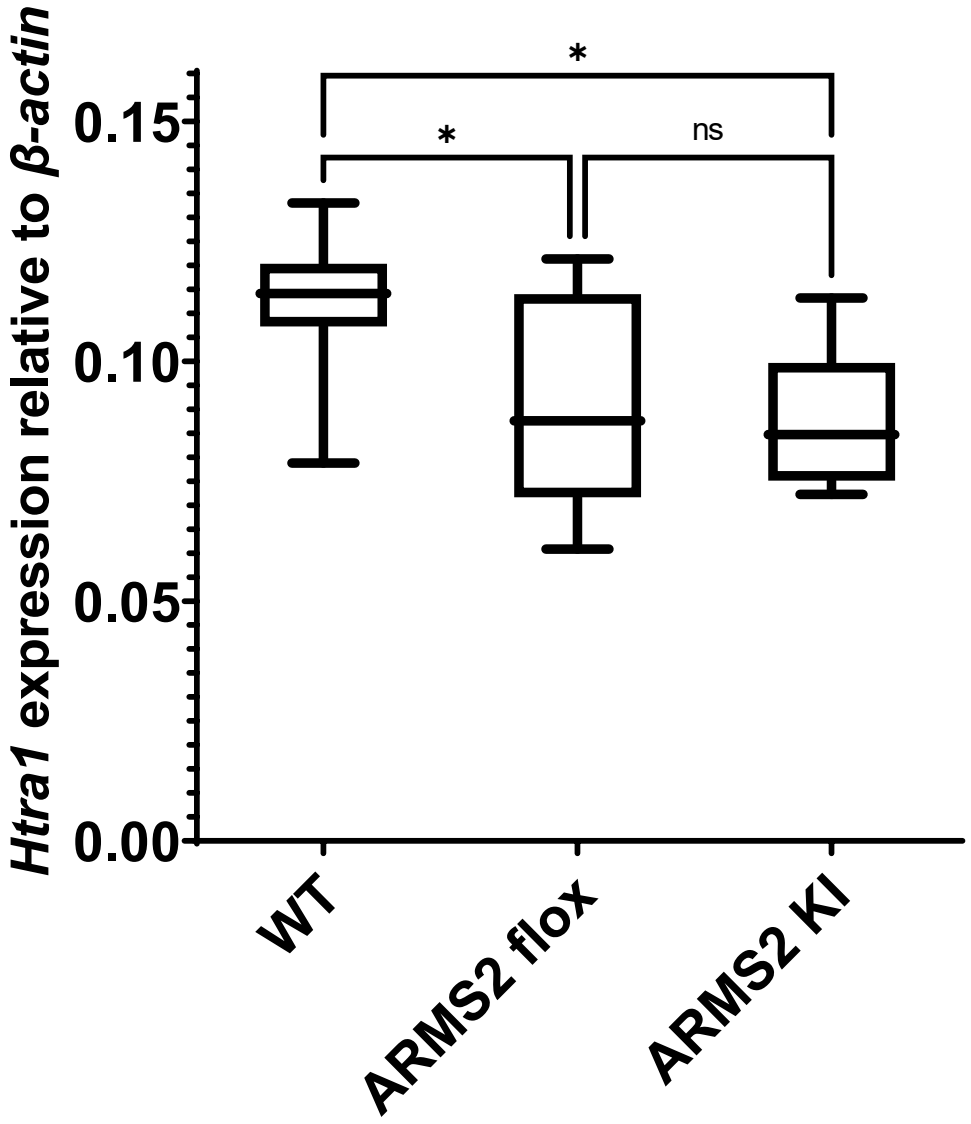

Figure 3 *Htra1* expression relative to *β-actin* in cortex

The boxs indicate upper and lower quartiles. The whiskers indicate the maximun and the minimum. * and ns indicate significant difference and nonsignificant difference in multiple comparisons (LSD), respectively. ANOVA: N=7 (WT), 7 (*ARMS2* flox), 9 (*ARMS2* KI).

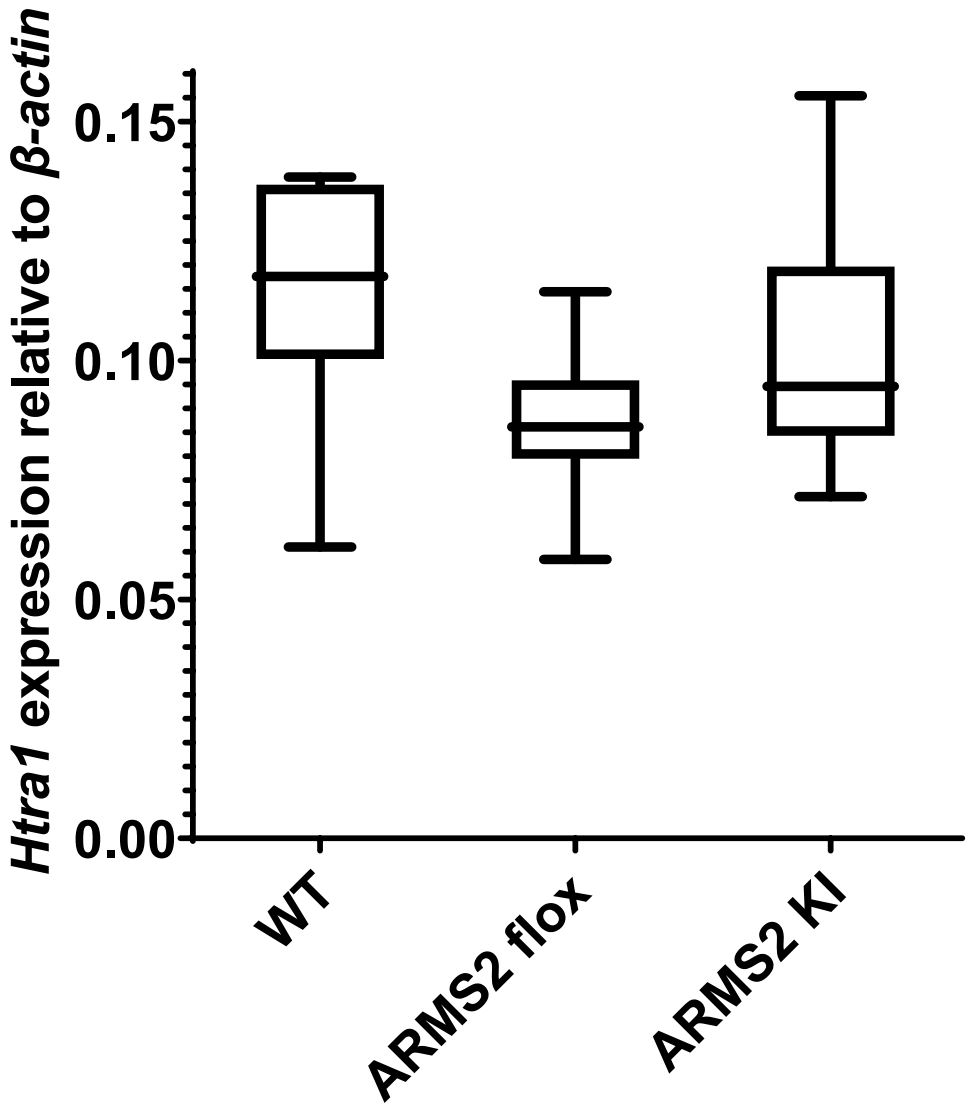

Figure 4 *Htra1* expression relative to *β-actin* in hippocampus

ANOVA: N=7 (WT), 7 (*ARMS2* flox), 9 (*ARMS2* KI).

Discussion

Although AMD is one leading cause of irreversible visual loss, the pathogenesis of it remains enigmatic till now. Genetic studies located several AMD susceptibility loci including two chromosome regions, 1q31 and 10q26. Evidence has emerged to support the association of AMD with each of the three candidate genes (later evidence pinpointed two genes, *ARMS2* and *HTRA1*) within 10q26. However, due to the existence of high LD across the region, statistical genetic analysis alone cannot definitively distinguish the causal one that confer the risk of AMD. Therefore, molecular functional studies became the better way to solve the puzzle. Little is known about the structure and function of ARMS2 protein. Several AMD-associated variants in *ARMS2* gene such as rs10490924, rs2736911 and the indel in 3' -UTR of *ARMS2* were identified, though how they might contribute to AMD pathogenesis remains inconsistent. More information is available for the function of HTRA1 protein: overexpressed HTRA1 has been hypothesized to digest excessive ECM and elevate oxidative stress to facilitate AMD pathogenesis. However, there is no consensus about how associated variants (such as rs11200638) might affect *HTRA1* expression. In summary, current evidence is inadequate to answer the causality question.

In order to provide insight of the functional roles of *ARMS2* and *HTRA1* in the pathogenesis of AMD, we decided to explore the regulation relationship between the two genes. To overcome the fact that *ARMS2* gene only exists in higher primates, we generated humanized *ARMS2* knock-in mice to investigate *Htra1* expression *in vivo*. *ARMS2* flox mice was also generated to distinguish whether the possible *Htra1* expression change derives from active *ARMS2* expression or genome structural change

upstream *Htra1*. Cortex and hippocampus were chosen for subsequent experiments. RNA was extracted and reverse-transcribed to cDNA. RT-qPCR was then performed to quantitate *Htra1* expression in different genotypes of mice.

We found significant difference of *Htra1* expression in cortex among three genotypes of mice. *Htra1* expression decreased by 22% and 18% for *ARMS2* KI mice and *ARMS2* flox mice, compared to wild type mice. Multiple comparisons also confirmed the significant decrease for both *ARMS2* KI mice and *ARMS2* flox mice but cannot distinguish these two genotypes of mice regarding their *Htra1* expression. This finding indicates that the presence of *ARMS2* gene upstream *Htra1* promoter manifests regulation effect upon *Htra1* expression in mouse model, also implying this relationship may exist in human. However, since we only observed *ARMS2* mRNA in *ARMS2* KI mice but not in *ARMS2* flox mice, we suspect this regulation relationship may not depend on the expression of *ARMS2* gene. Since *ARMS2* is quite closed to *Htra1* promoter region, *ARMS2* gene sequence itself may cooperate with *Htra1* promoter to regulate *Htra1* expression. Nevertheless, based on our data, we assume that *ARMS2* gene may negatively regulate *Htra1* gene expression. Thus, mutant variants which change the original sequence of *ARMS2* gene may distort the normal regulation relationship and lead to abnormal expression of *Htra1*. This hypothesis is partially consistent with previous evidence supporting that the indel variant in the 3'-UTR of *ARMS2* can enhance *Htra1* expression[24].

We didn't find significant difference of *Htra1* expression in hippocampus among three genotypes of mice, implying the regulation effect may present in a tissue-specific

manner, although individual variation among mice and relatively small sample size could be alternative explanations.

Yet the pathogenesis of AMD regarding the controversy of *ARMS2* and *HTRA1* remain unclear, a “dual causality hypothesis” which supports that both increased *HTRA1* and decreased *ARMS2* mediate AMD seem to be the answer suits for all observations [16][23]. Our findings provide new evidence of the functional link between *ARMS2* and *Htra1*, revealing the complexity of how the two genes might work together in the causal pathway of AMD. Future studies should consider the regulation effect when dissect the contribution towards AMD of one of the two genes.

The prominent strength of this study is the novel design of the humanized *ARMS2* knock-in mouse model. This construction solves the problem that higher-primate-specific gene *ARMS2* is intractable to investigate in model organisms, and will facilitate further research in this area. There are several limitations as well. First, we only chose early adult mice (18-20 day, postnatal) as our subjects. The fluctuation of *ARMS2* and *Htra1* expression in mice’s developmental process was ignored. Thus, we may miss the critical period when *ARMS2* and *Htra1* function in AMD pathogenesis. Second, we chose cortex and hippocampus based on our previous evidence such as *in situ* hybridization. The inconsistent result from cortex and hippocampus is hard to interpret, which reflects the logistic difficulty. To query the association with AMD pathogenesis, retinal tissue could be a better choice since retina is the major affected site of AMD. However, *ARMS2* is only faintly expressed in retina [18]. Our previous work didn’t detect *ARMS2* expression in retina either, which make the question hard to

tackle. Finally, the sample size is relatively small, which may let the true difference be covered up by the influence of individual variation of gene expression.

In the future, we could further design *ARMS2* knock-in mice carrying AMD risk variants such as rs10490924, rs2736911 and the indel in 3' -UTR of *ARMS2* to investigate their respective influence towards *Htra1* expression, and compare their effect with the original *ARMS2* allele. These comparisons could further validate the regulation relationship between *ARMS2* gene and *Htra1* gene. Besides, more elaborate detection of both ARMS2 protein and HTRA1 protein in eye tissue could be performed to ultimately ascertain the level of *ARMS2-Htra1* interplay.

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