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Characterization of the 10q26-orthologue in rhesus monkeys corroborates a functional connection between *ARMS2* and *HTRA1*

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ABSTRACT

Age-related macular degeneration, which is the leading cause of blindness in industrialized countries, is a multifactorial, degenerative disorder of the macula with strong heritability. For age-related macular degeneration in humans, the genes *ARMS2* and *HTRA1* in the region 10q26 are both promising candidates for being involved in pathogenesis. However, the associated variants are located in a region of strong linkage disequilibrium and so far, the identification of the causative gene in humans was not yet possible. This dilemma might be solved using an appropriate model organism. Rhesus monkeys suffer from drusen, a major hallmark of age-related macular degeneration, and the drusen-phenotype shares susceptibility factors with human macular degeneration. Thus, the rhesus monkey represents a natural animal model to uncover genetic factors leading to macular degeneration. Moreover, the existence of genetically homogenous cohorts offers an excellent opportunity to determine risk factors. However, the 10q26-orthologue genomic region in rhesus monkeys is not characterized in detail so far. Therefore, the aim of this study is to analyze the rhesus linkage disequilibrium structure and to investigate whether variants in *ARMS2* or *HTRA1* are associated with the drusen-phenotype as well. We sequenced parts of a 20 kb region around *ARMS2* and *HTRA1* in a genetically homogeneous cohort of 91 rhesus monkeys descending from the CPRC rhesus cohort on Cayo Santiago and currently housed in the German Primate Centre in Göttingen. Within this group, ophthalmoscopic examinations revealed a naturally high drusen prevalence of about 47% in monkeys >5 years. We detected 56 genetic variants within and around *ARMS2* and *HTRA1* and, as one deviates from Hardy-Weinberg-Equilibrium, 55 polymorphisms were used to generate a linkage disequilibrium-Plot and to perform association studies. We observed strong linkage disequilibrium between the markers and were able to define two haplotype blocks. One of these blocks spanned the whole *ARMS2* locus and the 5′ part of *HTRA1* – almost perfectly resembling the situation found in humans. Tests for association revealed a variant in the promoter region of *HTRA1* and two variants in the 5′-UTR of *ARMS2* to be associated with drusen. The strong linkage disequilibrium inhibits – as in humans – a determination of the risk gene using statistical methods only. However, the conserved linkage disequilibrium structure in humans and macaques goes in line with the recently emerged dual causality model proposing that *ARMS2* and *HTRA1* are functionally connected and that both genes contribute to the disease pathology. Moreover, the characterization of the 10q26-orthologue genomic region of the rhesus monkey provides a basis for now needed functional investigations in a well-characterized model organism.

1. Introduction

Age-related macular degeneration (AMD) is a degenerative disorder of the macula and the leading cause of blindness in industrialized countries (Schrader, 2006). It is a complex disease caused by an interaction of environmental and genetical risk factors, whereby heritability is estimated to make up to 71% (Scholl

Abbreviations: AMD, age-related macular degeneration; LD, linkage disequilibrium; PCR, polymerase-chain-reaction; ARMS2, AMD-related maculopathy susceptibility 2; HTRA1, high-temperature requirement factor A1; CFH, complement factor H; OR, odds ratio; CEU, Utah residents with northern and western European Ancestry.

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et al., 2007). In genome-wide association studies several candidate regions have been identified and the *CFH*-gene on 1q13 as well as the region 10q26 containing the genes *ARMS2* and *HTRA1* are discussed as major susceptibility loci (Jakobsdottir et al., 2005; Rivera et al., 2005). However, *ARMS2* and parts of *HTRA1* are located in a region of strong linkage disequilibrium (LD) (Fritsche et al., 2008) and, thus far, in humans the determination of the gene playing a role in AMD-pathogenesis was not possible (Friedrich et al., 2011). Functional assays provide contradictory results (An et al., 2010; Kanda et al., 2010; Wang et al., 2010; Chan et al., 2007; Dewan et al., 2006) Table 1.

Analyzing the situation in rhesus monkeys – known to be a good model organism for macula degeneration (Hope et al., 1992; El-Mofty et al., 1978) – might help solving this dilemma. Rhesus monkeys develop an age-related drusenoid maculopathy comparable to human AMD (Gouras et al., 2008) and also share genetic susceptibility factors (Francis et al., 2008). Moreover, the existence of genetically homogenous cohorts offers an excellent opportunity to identify drusen-specific risk factors. It has been shown that variants in the rhesus 10q26-orthologue on chromosome 9 are associated with drusen formation as well (Singh et al., 2009; Francis et al., 2008). However, to our best knowledge, no comprehensive characterization of the candidate region in the rhesus monkey exists. For the first time, this study determines the linkage disequilibrium structure of the 10q26-orthologue genomic region in rhesus monkeys. Furthermore, we investigated in an explorative approach whether rhesus variants in *ARMS2* or *HTRA1* are associated with the drusen-phenotype.

2. Materials and methods

2.1. Animals

The study group comprised 43 rhesus monkeys affected with drusen and 48 healthy controls. The 91 animals were aged 5–25 years and are housed in the German PrimateCenter in Göttingen. 79 of these originated from the CPRC rhesus cohort on Cayo Santiago and 12 were of different origin. The history of the Cayo Santiago colony is described elsewhere (Singh et al., 2005). During the annual health check, EDTA blood was obtained and ophthalmoscopic analysis of both eyes was performed using a fundus camera (Abott, Germany). All procedures involving the rhesus monkeys were approved by local authority and carried out in accordance with the EC Directive 86/609/EEC for animal experiments.

2.2. Genotyping

Genomic DNA was extracted from EDTA blood using *QIAamp DNA Mini Kit* (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The sequence around ARMS2 and HTRA1 was downloaded from the UCSC Genome Browser (http://genome.ucsc.edu/index.html) that automatically highlights repetitive regions by using the latest version of the RepeatMasker (http://www.repeatmasker.org/). The region is enriched with repetitive elements and to obtain reproducible results as well as informative marker, only fragments without such repetitive elements were chosen for further analysis. The sequence data including the location of 14 fragments together with the respective primers were provided by the Institute of Human Genetics in Regensburg. To cover all main areas without repetitive elements, seven further fragments were added by us, so that altogether 21 fragments distributed in a 20 kb region up- and downstream of *ARMS2* and *HTRA1* were chosen to screen 8 affected animals for suitable polymorphisms. Mostly, 30 ng genomic DNA, 10 μM forward and reverse Primer, 1× Taq polymerase buffer, 1 U Taq polymerase, 200 μM of each dNTP and – if necessary – 1xQ-solution and additional $MgCl_2$ (1.5 mM) were used for amplification. Polymerase chain reaction (PCR) was performed under standard conditions consisting of 32 cycles of denaturation at 95 °C for 60 s, annealing at the respective adapted temperature for 60 s and extension at 72 °C for 60 s in 96-well plates in the PCR thermocycler TProfessional (Biometra, Germany). For some fragments, a Hot Start PCR using 30 ng genomic DNA, 10 μM forward and reverse Primer, 1× Phire polymerase buffer, 1 U Phire polymerase and 200 μM dNTPs (each) was alternatively performed. Hot Start PCR conditions consisted of 25 cycles of 5 s denaturation at 98 °C, 5 s annealing at the adapted temperature and 20 s elongation at 72 °C. For both PCR types, an initial denaturation step and a final elongation step were performed. For annealing temperatures, primer sequences and further details see supplementary Table 1. The PCR products were purified with ExoSAP-IT (USB Cooperation, Cleveland, OH, USA) and sequenced with 1 μM of primer (forward and reverse) using the BigDye Terminator Cycle Sequencing Kit v.1.1 (Applied Biosystems, Darmstadt, Germany) according to the manual. For PCR products longer than 650 bp two additional internal primers were used. The products were purified with Sephadex® G-50 (Sigma–Aldrich Chemie GmbH, Munich, Germany) plates and analysed with the ABI Prism™ Genetic Analyser 3110xl.

In a further step, the identified variants were genotyped in all animals by either sequencing, pyrosequencing or with restriction fragment length analysis. Sequencing was performed as previously described. For pyrosequencing, PCRs with one biotinylated primer were performed under standard conditions. Pyrosequencing was conducted using the *Pyro Gold CDT Kit*, the *PyroMark Vacuum Prep Workstation* and the *PyroMark MD* from Qiagen (Qiagen GmbH; Hilden, Germany) according to the supplier's instructions. For the polymorphism genotyped by restriction fragment length analysis, the PCR products were digested with Tsp509I and electrophoresed on a 3% NuSieve/1%Seakem agarose (FMC BioProducts, Rockland, ME) gel. Primer sequences, PCR product sizes and other assay details for these assays are listed insupplementary Table 2.

2.3. Statistical analysis

Hardy–Weinberg equilibrium was assessed for cases and controls using *PLINK* (http://pngu.mgh.harvard.edu/~purcell/plink/, Purcell et al., 2007). For the explorative SNP association analysis, we applied the Cochran-Armitage trend test followed by a logistic regression analysis of the positive variants. Thereby, one analysis was performed without covariates and a second analysis was adjusted for origin. Both analyses modelled an additive genetic effect.

The construction of the LD plots was performed using *Haploview 4.1* (www.broad.mit.edu/mpg/haploview/, Barrett et al., 2005). All rhesus monkey variants that were in HWE were used for LD-plot-generation. The human LD-Plot was constructed with the HapMap data (Version 2, release 3, population: CEU) for the orthologous region (Chr10: 124,190,381–124,235,021). Haplotype analysis of the associated variants was conducted with *Haploview 4.1* as well

Table 1
Results of the tests for association performed with variants within the LD region around *ARMS2* and *HTRA1*.

ss#	Cochran-Armitage trend test*	Logistic regression*	
		p	*p* adjusted for origin
290433540	0.04614	0.04980	0.25070
290433541	0.04970	0.05341	0.34780
290433555	0.01519	0.01785	0.05728

*p-value with $p < 0.05$ considered as statistically significant.