Synonymous variants in HTRA1 implicated in AMD susceptibility impair its capacity to regulate TGF-β signaling

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Abstract

High-temperature requirement A1 (HTRA1) is a secreted serine protease reported to play a role in the development of several cancers and neurodegenerative diseases. Still, the mechanism underlying the disease processes largely remains undetermined. In age-related macular degeneration (AMD), a common cause of vision impairment and blindness in industrialized societies, two synonymous polymorphisms (rs1049331:C>T, and rs2293870:G>T) in exon 1 of the HTRA1 gene were associated with a high risk to develop disease. Here, we show that the two polymorphisms result in a protein with altered thermophoretic properties upon heat-induced unfolding, trypsin accessibility and secretion behavior, suggesting unique structural features of the AMD-risk-associated HTRA1 protein. Applying MicroScale Thermophoresis and protease digestion analysis, we demonstrate direct binding and proteolysis of transforming growth factor β1 (TGF-β1) by normal HTRA1 but not the AMD-risk-associated isoform. As a consequence, both HTRA1 isoforms strongly differed in their ability to control TGF-β mediated signaling, as revealed by reporter assays targeting the TGF-β1-induced serpin peptidase inhibitor (SERPINE1, alias PAI-1) promoter. In addition, structurally altered HTRA1 led to an impaired autocrine TGF-β signaling in microglia, as measured by a strong down-regulation of downstream effectors of the TGF-β cascade such as phosphorylated SMAD2 and PAI-1 expression. Taken together, our findings demonstrate the effects of two synonymous HTRA1 variants on protein structure and protein interaction with TGF-β1. As a consequence, this leads to an impairment of TGF-β signaling and microglial regulation. Functional implications of the altered properties on AMD pathogenesis remain to be clarified.

†The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

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Introduction

The high-temperature requirement A (HTRA) family of proteins is broadly characterized by a highly conserved protease domain and one or more C-terminal PDZ protein-interaction domains (1–9). One of its members, HTRA1, is a secreted protease with an extracellular export signal, a Mac25/IGFBP domain, a Kazal-like structure, a serine protease module and a single C-terminal PDZ binding motif (3,4). Functionally, HTRA1 is thought to be involved in a number of biological processes and was reported to influence cell signaling (5–9), organization of the extracellular matrix (10–13) and skeletal development and osteogenesis (14,15).

In addition, HTRA1 was suggested to influence several disease-associated processes, particularly in CARASIL (cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy) (16,17), osteoarthritic cartilage (10,11,18), preeclampsia (19) and choroidal neovascularization (9,13,20). HTRA1 may also mediate tumor suppressor activities (21–23), as down-regulation of the protein was observed in a variety of cancers, e.g. endometrial, ovarian or breast cancers, and melanomas (21,22,24–27). Despite its suspected role in the various diseases, the exact mechanisms of HTRA1 action are largely unknown.

DNA variants in the coding region of the HTRA1 gene have been associated with two diseases including the autosomal dominant CARASIL (16,28–32) and the complex age-related macular degeneration (AMD) (33,34). For CARASIL, HTRA1 mutations in conserved regions of its protease domain or deleterious nonsense mutations were suggested to underlie disease pathogenesis by impairing control of HTRA1 over TGF-β-regulated pathways thus increasing levels of TGF-β in cerebral small arteries (16). In accordance with these results, HTRA1-deficient mice exhibited increased levels of phosphorylated SMAD proteins, downstream effectors of the TGF-β signaling cascade in brain tissue (9).

Several independent case–control association studies have revealed a strong association of AMD pathology with two variants located in exon 1 of the HTRA1 gene (rs1049331:C>T, and rs2293870:G>T) (6,35–37). Although synonymous, these variants were recently shown to directly influence the ability of HTRA1 to regulate insulin-like growth factor 1 (IGF1) signaling (6). However, the binding capacity of HTRA1 for IGF1 is controversial (4).

Here, we have reanalyzed HTRA1 variants rs1049331:C>T and rs2293870:G>T by independent methodological approaches for their effects on protein structure. In our analysis, we have also included a third variant, rs2293870:G>C, which is located at the same nucleotide position as rs2293870:G>T but is not associated with AMD disease. By MicroScale Thermophoresis (MST), we demonstrate an influence of the AMD-risk-associated polymorphisms rs1049331:C>T and rs2293870:G>T on HTRA1 protein folding but not for rs2293870:G>C. We also show that the two HTRA1 protein isoforms reveal differences in their ability to regulate TGF-β signaling. Specifically, the HTRA1 isoform translated from risk variants rs1049331:T and rs2293870:T binds less efficiently to TGF-β and is less proteolytically active. As a consequence, control over TGF-β-regulated pathways becomes impaired although a role of these processes in AMD pathogenesis remains to be shown.

Results

Statistical evaluation of tri-allelic variant rs2293870 for AMD risk association

We evaluated the association of late-stage AMD risk with G, T and C alleles at the tri-allelic rs2293870 variant located in the exon 1 coding sequence of the HTRA1 gene. With rs2672601:A, a suitable biallelic proxy variant for the rs2293870:C allele was identified in 503 Europeans (r² to the C allele of rs2293870 of 0.96; 1000 Genomes project, Phase 3). We then genotyped rs2672601 (for the A allele) and rs2293870 (for the C and T allele) in 1,667 late-stage AMD cases and 1,148 AMD-free controls. Subsequently, we fitted a multivariate logistic regression model including both rs2293870 and rs2672601. In agreement with previous reports (6,35–37), we found that the T allele of rs2293870 is strongly associated with AMD (odds ratio [OR] 2.98, 95% confidence interval [CI] 2.62–3.40, P < 1 x 10exp-16). On the other hand, rs2672601 (and thus the C allele of rs2293870) revealed no association with late stage AMD (OR 0.94, 95% CI 0.79–1.10, P = 0.41). The C allele of rs1049331 is on the same haplotype as the G and C allele of rs2293870 (non-AMD associated), and the T allele of rs1049331 is on the same haplotype as the T allele of rs2293870 (AMD associated). We observed no discordant haplotypes in 503 Europeans from the 1000G project.

Synonymous DNA variants rs1049331:C-T and rs2293870:G-T but not rs2293870:G>C in exon 1 of HTRA1 alter the conformation of the secreted protein

Three expression constructs for HTRA1 were generated including HTRA1 exon 1 variants rs1049331:C and rs2293870:G [reference haplotype (36), referred to as HTRA1:CG], the AMD-associated variants rs1049331:T and rs2293870:T [HTRA1:TT], and the non-disease-associated variants rs1049331:C and rs2293870:C (HTRA1:CC) (Fig.1A). To assess an influence of codon usage on structural properties, we heterologously expressed the different HTRA1 variants in human Hek293 cells. Owing to the mammalian expression system and to auto-proteolytic activity of HTRA1, protein yield was low rendering several methods for structural analysis inappropriate. In addition, HTRA1 contains no tryptophan residue in its amino acid sequence, limiting the application of fluorometric methods. To assess protein folding properties of the translation products, we first applied MST, a method which determines the movement of molecules along a temperature gradient (38,39). The thermophoretic movement is very sensitive to changes of the molecular structure or conformation. To specifically detect alterations of the structure of the HTRA1 isoforms, the thermophoretic movement was determined under different temperature conditions (from 32 to 52°C in 2°C steps). The protein was fluorescently labeled at the C-terminus via its tetra-cysteine(TC) tag (40,41).

MST analysis of the three HTRA1 constructs at different temperatures revealed a similar thermal migration behavior of the two HTRA1 isoforms HTRA1:CG and HTRA1:CC (Fig. 1B). In contrast, HTRA1:TT exhibited a significantly different thermophoretic mobility at higher temperatures (Fig. 1B) suggesting an influence of AMD-associated polymorphisms rs1049331:T and rs2293870:T on the tertiary structure of the protein.

In an independent approach, we assessed structural differences of the three protein isoforms by partial proteolysis. This assay is sensitive to minor alterations in protein structure by measuring the susceptibility of the protein substrate for proteases such as trypsin (42–45). This protease susceptibility of a protein is strongly dependent on unfolding processes and, in contrast to fluorometric detection systems, the proteases detect local and not mainly global unfolding (44). Under partial denaturing conditions, HTRA1:TT was more susceptible to trypsin digestion than HTRA1:CG or HTRA1:CC (Fig. 1C). Specifically, after preincubation at 42°C, a strong decrease of HTRA1:TT was observed, and after preincubation at 46°C, HTRA1:TT was completely digested (Fig. 1D). In contrast, no significant reduction of
HTRA1:CG and HTRA1:CC, subjected to these conditions, was observed. The differences in protein levels between HTRA1:CG or HTRA1:CC and HTRA1:TT were statistically significant after preincubation at 42°C (P < 0.05), and highly significant after preincubation at 46°C (P < 0.01). Autoproteolysis of HTRA1 was negligible (Fig. 1C).

Together, the results from MST analysis and from partial proteolysis suggest that HTRA1:CG and HTRA1:CC exhibit a similar tertiary structure that is different from HTRA1:TT. With a further focus on the functional relevance of the altered structure of HTRA1:TT, following we concentrated on this variant and compared its properties with the HTRA1 isoform translated from the reference haplotype, namely HTRA1:CG.

**Delayed secretion of HTRA1 protein isoform HTRA1:TT in Hek293 cells**

Secreted proteins are subjected to ER quality control, which retains and eventually degrades misfolded protein species with a high sensitivity toward minor structural alterations (46). We therefore monitored secretion of HTRA1:CG and HTRA1:TT in Hek293 cells. The supernatant and cells were collected at various time points between 0 and 24 h after transfection with expression constructs for HTRA1:CG and HTRA1:TT (Fig. 2). Western blot analysis was performed with α-HTRA1 antibody. Relative to HTRA1:CG, intracellular amounts of HTRA1:TT were increased, with a statistically significant difference at 24 h (P < 0.05) (Fig. 2A and B). Conversely, a reduction of HTRA1:TT protein relative to HTRA1:CG was detected in the supernatant (Fig. 2A and C). At time points 20 and 24 h, the differences in the amounts of extracellular protein of HTRA1:CG and HTRA1:TT were statistically significant (P < 0.05) (Fig. 2C). These data suggest a delayed secretion of HTRA1:TT relative to HTRA1:CG.

**HTRA1 protein isoform HTRA1:TT reveals significantly reduced TGF-β1 binding and proteolysis**

Several studies demonstrated a negative, inhibiting influence of HTRA1 on TGF-β signaling, although the type of interaction between the two molecules remained controversial (5,7,9,17,47,48). To investigate the HTRA1–TGF-β interplay, we used MST analysis and measured thermophoretic mobility of HTRA1 dependent on...
HTRA1 isoform HTRA1:TT exhibits a reduced capacity for TGF-β signaling inhibition

To examine whether the altered affinity of TGF-β1 and HTRA1:TT affects TGF-β signaling, we used a reporter assay based on MLEC-PAI1Luc cells (49). These cells are stably transfected with a luciferase coding sequence under the control of the TGF-β responsive element of the PAI-1 promoter and thus respond to stimulation by TGF-β family members (e.g. TGF-β1) with heterologous luciferase expression (49). Addition of HTRA1:CG to MLEC-PAI1Luc cells resulted in a strong reduction of TGF-β1-activated luciferase expression (17.1 ± 3.6% luciferase activity compared with control cells). HTRA1:TT was also capable of inhibiting TGF-β1-activated luciferase expression, but only at 67.0 ± 4.5% (Fig. 4). The difference in luciferase expression between cells treated with HTRA1:CG and HTRA1:TT was statistically highly significant (P < 0.01).

HTRA1 isoform HTRA1:TT exhibits reduced control over autocrine TGF-β signaling in microglial cells

TGF-β signaling has been reported to be an important regulator of microglial activation (50–53). Specifically, extracellular binding of TGF-β family members by TGF-β receptors leads to phosphorylation of SMAD proteins (54–56). We therefore analyzed the influence of HTRA1:CG and HTRA1:TT on the phosphorylation of SMAD2 in BV-2 cells (Fig. 5). Immunocytochemistry with control cells showed a strong immunoreactivity for phosphorylated SMAD2 (pSMAD2) within BV-2 cells, indicating an activated TGF-β signaling pathway by endogenously synthesized and secreted TGF-β1 proteins (Fig. 5A, left), in full agreement with data on autocrine TGF-β expression and signaling in microglial cells (57). BV-2 cells treated with HTRA1:CG exhibited a significantly reduced signal intensity of pSMAD2 indicating an inhibiting effect of HTRA1:CG upon TGF-β signaling (Fig. 5A, middle). In contrast, staining for pSMAD2 in BV-2 cells treated with HTRA1:TT had a similar intensity as in the control experiment (Fig. 5A, right). To quantify the effects of HTRA1 isoforms on SMAD phosphorylation, we performed western blot analyses and densitometry (Fig. 5B and C). The data show that SMAD2 phosphorylation is almost completely absent in BV-2 cells treated with HTRA1:CG whereas HTRA1:TT reduced measurable pSMAD2 levels to 71.9 ± 2.5% compared with control (Fig. 5B and C). While the amounts in SMAD2 protein were comparable, the observed differences in phosphorylated pSMAD2 were statistically highly significant (P < 0.01) (Fig. 5C).

Upon TGF-β stimulation, phosphorylated SMAD2 and SMAD3 form a complex with SMAD4, which is then translocated into the nucleus and stimulates expression of TGF-β response genes (54–56). After treating BV-2 cells with HTRA1:CG and HTRA1:TT for 3 and 24 h, we monitored the expression of PAI-1, a prominent target gene of TGF-β signaling (58–60) (Fig. 5D). Upon quantitative RT-PCR, a strong down-regulation of PAI-1 mRNA expression was noted in BV-2 cells treated with HTRA1:CG when compared with control cells (12.5 ± 12.9% after 3 h; 14.5 ± 13.9% after 24 h). In contrast, a less prominent decrease of PAI-1 transcripts was found after treatment with HTRA1:TT (74.0 ± 35.5% after 3 h; 56.8 ± 29.6% after 24 h, compared with control cells). The difference in PAI-1 expression following treatment with HTRA1:CG or HTRA1 TT was statistically significant (P < 0.05).

Discussion

The present study focused on the impact of three synonymous SNPs (rs1049331:C>T, rs2293870:G>T and rs2293870:G>C) on...
HTRA1 structure and function. We first re-analyzed AMD association of these three SNPs located in coding exon 1 of the HTRA1 gene in nearly 3000 individuals with and without AMD disease. In agreement with earlier reports (6,35–37), we show that the rs2293870:T allele is indeed highly associated with AMD whereas genotyping a perfect proxy for the rs2293870:C allele (rs2672601:A) revealed no association with disease risk. We now provide evidence that the rs1049331:T/rs2293870:T haplotype (HTRA1:TT) significantly alters HTRA1 folding and the ability of the protein to bind TGF-β1. The third SNP (rs2293870:G>C) despite affecting the identical nucleotide position as rs2293870:G>T revealed functional features comparable with the reference sequence (rs1049331:C, rs2293870:G or HTRA1:CG).

In recent years, the influence of synonymous, silent mutations on protein folding and function has attracted increasing attention (61). These alterations although not associated with changes in amino acid composition of the protein were reported to cause mRNA instability, exon skipping or alterations in co-translational protein folding (43,62–70). As a consequence, synonymous alterations in protein-coding sequences may causally be involved in human pathologies. For example, synonymous changes in the multidrug resistance 1 (MDR1) gene were shown to cause an altered drug and inhibitor interaction leading to major changes in protein stability and transporter specificity (43,71).

Figure 3. Influence of synonymous SNPs within HTRA1 exon 1 on its substrate affinity. (A) HTRA1 and its interaction with TGF-β1 as measured by MST. Fluorescence-labeled HTRA1 (100 nm) was incubated with increasing concentrations of TGF-β1 (0.122–4000 nM). (B) HTRA1 and its interaction with β-Casein as measured by MST. Fluorescence-labeled HTRA1 (100 nm) was incubated with increasing concentrations of β-Casein (1.22–40000 nM). Protein-protein interactions for (A) and (B) were quantified by MST and binding data were plotted applying the Hill equation. The recorded fluorescence was normalized to fraction bound (0 = unbound, 1 = bound). Data represent the mean ± SD of three independent experiments. (C) Proteolysis of TGF-β1 by HTRA1:CG, HTRA1:TT or control. One microgram of TGF-β1 was mixed with 300 μl supernatant of transfected Hek293 cells, containing 15 ng/μl of each HTRA1 isoform, and incubated at 37°C. Samples were taken at the indicated time points. TGF-β1 proteolysis was captured via western blot analysis with α-TGF-β antibody. (D) Three independent experiments were performed for TGF-β1 as shown in (C) and subjected to densitometrical analysis. TGF-β1 signals were calibrated against measurements for HTRA1:CG at 0 h. Data represent the mean ± SD. Asterisks mark statistically significant (**P<0.05) and highly significant differences (****P<0.01) between TGF-β1 cleavage by HTRA1:CG and HTRA1:TT, respectively. (E) Proteolysis of β-Casein by HTRA1:CG, HTRA1:TT or control. Twenty micrograms of β-Casein were mixed with 300 μl supernatant of transfected Hek293 cells, containing 10 ng/μl of each HTRA1 isoform, and incubated at 37°C. β-Casein was visualized by Coomassie staining. (F) Three independent experiments were performed for β-Casein as shown in (E) and subjected to densitometrical analysis. β-Casein signals were calibrated against measurements for HTRA1:CG at 0 h. Data represent the mean ± SD.
observed direct proteolysis of mature TGF-β but not cleave mature TGF-β. The precise mechanism of HTRA1 interference with this pathway is still contested. Initially, HTRA1 was shown to bind to but not cleave mature TGF-β proteins whereas other studies observed direct proteolysis of mature TGF-β family members by HTRA1. Afterward, the latter findings were refuted by Shiga and colleagues, who described proteolysis by HTRA1 only for the intracellular pre-form of TGF-β (17). Similarly, Graham and colleagues also could not find an interaction of HTRA1 and TGF-β but instead described cleavage of TGF-β receptors by HTRA1. Applying MST and TGF-β digestion analyses, we provide evidence for a direct proteolytic activity of isoform HTRA1:CG toward mature TGF-β1 in agreement with earlier reports (9,47). Nevertheless, we cannot exclude other so far unknown sites of interaction at which HTRA1 antagonizes the TGF-β pathway.

Interestingly, the MST data presented here revealed no stable interaction between HTRA1:TT and TGF-β1, whereas the TGF-β1 in vitro digestion assay suggested a strongly decreased turnover rate of TGF-β1 by HTRA1:TT compared with HTRA1:CG. The apparent discrepancy raises the question why there is still an interaction of TGF-β1 and HTRA1 in the proteolysis assay, whereas at the same time, no interaction of the two proteins is apparent in the MST assay. We hypothesize that MST measures a stable interaction between HTRA1 and TGF-β1, resulting in a binding constant whereas the proteolytic assay measures enzyme kinetics over a longer period of time. A stable interaction between protease and substrate is not necessary for proteolytic cleavage, as described e.g. for rhomboid proteases (73). These proteases displayed no physiological affinity for their substrates. Instead, an approximate 10 000-fold difference in proteolytic efficiency with substrate mutants and diverse rhomboid proteases were reflected in kcat values alone.

The reduced proteolytic capacity of HTRA1:TT for TGF-β1 severely affected the regulation of TGF-β1 signaling by HTRA1, as observed in reporter assays assessing the PAI-1 promoter, a prominent target of the TGF-β cascade. In agreement with this, Hara and colleagues (16) reported similar consequences owing to CARASIL-associated HTRA1 variants. Specifically, two nonsense mutations and one missense mutation in HTRA1 resulted in protein products that failed to repress signaling by the TGF-β family (16). As increased TGF-β levels were observed in cerebral arteries of CARASIL patients, the authors concluded that HTRA1 contributes to the pathogenic processes of CARASIL via its control of TGF-β signaling. In this context, it is interesting to note that increased TGF-β levels are also observed in AMD patients (74,75) and aberrant TGF-β signaling was connected to AMD-related phenotypes in mice (9,76,77) and RPE cells (77–80). This and our findings could imply a role for HTRA1 in AMD pathogenesis via its regulation of the TGF-β pathway.

We also demonstrated control of HTRA1 over TGF-β regulation in cultured microglial cells. Microglia are the major resident inflammatory cells of the central nervous system and obtain key functions of immune surveillance and tissue repair (81–83). Thus, this cell type is deeply involved in the pathogenic processes of various neurodegenerative diseases like Parkinson’s disease (84), Alzheimer’s disease (85–89), inherited retinopathies (90) or AMD (91–95). In addition, activation and differentiation of microglia is strongly regulated by TGF-β signaling (96–99). We now showed that pSMAD2 and PAI-1 expression, both markers for an activated TGF-β signaling (54–56,58–60), were significantly lower in microglial (BV-2) cells treated with HTRA1. Thus, our data further provide evidence for a role of HTRA1 in the differentiation and activation of microglia, and therefore likely in the pathogenesis of neurodegenerative disease. Of note, the inhibitory effect of HTRA1:TT on microglial TGF-β1 signaling was weaker compared with the reference HTRA1 isoform, HTRA1:CG.

Taken together, our data show that HTRA1 is an important regulator of TGF-β signaling by direct interaction with and cleavage of TGF-β. AMD-associated polymorphisms in HTRA1 (rs1049331:6366 | Human Molecular Genetics, 2015, Vol. 24, No. 22

![Figure 4](image-url)

**Figure 4.** Effects of HTRA1:CG and HTRA1:TT on TGF-β1-induced PAI-1 promoter activity in MLEC(PAI/Luc) cells. MLEC(PAI/Luc) cells were stimulated with 2 ng/ml TGF-β1 in the presence of 40 ng/ml HTRA1:CG or HTRA1:TT, which were purified from supernatant of HeK293 cells transfected with expression constructs for Strep-tagged HTRA1, or control protein (purified from supernatant of empty Strep-vector transfected cells). After 16 h, luciferase activity was measured in triplicate wells each in three independent experiments. Measurements for each experiment were calibrated against the control (TGF-β1 + control protein). The mean ± SD for the three independent experiments is given for each treatment. Asterisks mark statistically highly significant differences (P<0.01).

altered only for the protein isoform encoded by the transcript harboring the synonymous variants rs1049331:T and rs2293870: T (HTRA1:TT), but not for the other isoforms, harboring rs1049331:C and rs2293870:G (HTRA1:CG), or rs1049331:C and rs2293870:C (HTRA1:CC). Also, we provide evidence for a decreased secretion of the AMD-associated HTRA1 variant over the reference isoform. The impact of the synonymous nucleotide changes at HTRA1:CG, HTRA1:TT and HTRA1:CC on its protein structures remains to be determined. They localize to the N-terminal domain of HTRA1, known as the IGFBP-like module, which exhibits a similar fold as the N-terminal lobe of the IGF-binding protein (4). This domain apparently has no influence on folding or protease activity of the HTRA1 C-terminus (4) but could mediate protein–protein interactions (6). This suggests that a structural alteration of this domain could alter substrate binding capacities of HTRA1.

Our results are in full agreement with findings first reported by Jacobo and colleagues (6). In their work, the authors observed a decreased translation rate of the isoform HTRA1:TT which they ascribed to differential codon usage (6). They also reported differential susceptibilities of this variant toward proteolysis, an anti-HTRA1 antibody and IGF-1 interaction. However, the interaction between HTRA1 and IGF-1 is challenged by findings from Eigenbrot and colleagues (4) who failed to obtain evidence for a binding of IGF-1, IGF-II or insulin binding activity for the N-domain of HTRA1. This is further supported by a biosensor binding study which found no appreciable Mac-25 interaction of HTRA1 with IGF-1 or IGF-II (72).

We therefore focused on the widely reported influence of HTRA1 on TGF-β signaling (5,7,9,17,47,48). Although generally accepted, the precise mechanism of HTRA1 interference with this pathway is still contested. Initially, HTRA1 was shown to bind to but not cleave mature TGF-β proteins whereas other studies observed direct proteolysis of mature TGF-β family members by
**Materials and Methods**

**Genotyping the C-allele of rs2293870 and statistical evaluation**

Owing to the tri-allelic nature of the variant rs2293870, direct genotyping is challenging. We therefore searched for a suitable biallelic proxy variant for the C-allele of rs2293870 in 503 European individuals from the 1000 Genomes project [allele frequency in Europeans: 0.16, Phase 3, accessed July 2015 (101)]. The biallelic variation rs2672601 (G>A, allele frequency of A in Europeans: 0.16) is in high linkage disequilibrium with rs2293870 ($R^2 > 0.96$, $D' = 1$), and the A allele of this variation was used as a surrogate for rs2293870:C. We genotyped a total of 2,815 individuals as previously described (102) and coded the genotype at rs2672601 as the number of A alleles (0, 1 or 2) and at rs2293870 as the number of T (risk associated) alleles (0, 1 or 2). Multivariate logistic regression implemented in R (103) was used to evaluate the association of the A allele of rs2672601 and T allele of rs2293870 with AMD risk.

**Expression cloning**

The coding sequences of three HTRA1 haplotypes were heterologously expressed in Hek2893 cells. These included the most frequent HTRA1 haplotype (HTRA1:CG), as well as two less common HTRA1 haplotypes (HTRA1:TT and HTRA1:CC) (Fig. 1A). After genotyping, the respective coding sequences were amplified from cDNA of retinal tissue as described (100). For MST analyses, the three HTRA1 haplotypes were each tagged with a C-terminal tetra-cysteine (TC) tag [5' TGT TGT CCT GCC TGT TGC 3'] and cloned into the NotI/XhoI site of the pCEP4 vector (Invitrogen, Carlsbad, CA). Oligonucleotide primers for amplification were designed to contain a NotI site 5' of the HTRA1 translational start site (HTRA1-NotI-F, 5' GCG GCC CGG CGC ACT CGC ACC CGC T 3') and a TC tag followed by an XhoI...
Site at the 3′-end of the HTRA1-coding sequence (HTRA1-TC-Xhol-R, 5′ GCA ACA GCC AGG ACA TGG GTG CAT ATT TTG TTC GGG AAT CAC TGT GAT CTC GAG CTA GCA ACA GCC AGG ACA ACG TGG CAT ATT TTG TT 3′).

For purification, coding sequences of HTRA1:CG, HTRA1:TT and HTRA1:CC were cloned into the BamHI/Xhol site of the pEXPR-IBA103 vector (IBA Life Sciences) fusing the insert to a labeled in cell culture medium with 5 μM FlAsH-EDT2.

For MST analysis, TC-tagged HTRA1 proteins (2 μg/μl each) were harvested 24 h after transfection, cell culture medium was changed to serum-free medium.

For sodium dodecyl sulfate–polyacrylamide gel electrophoresis, western blot analysis and Coomassie staining

For sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), Laemmli Buffer (105) was added to the samples and the proteins were separated on 15% gels. For western blotting, proteins were transferred to polyvinylidene fluoride membranes (Immobilon, Milipore, Schwalbach, Germany), or BSA (AppliChem GmbH, Darmstadt, Germany, only for TGF-β1 detection) for 1 h. Subsequently, membranes were incubated with primary antibodies in PBS overnight at 4°C. Primary antibodies were obtained commercially as follows: HTRA1 (ab65902; rabbit polyclonal, application as a 1:2000 dilution, Abcam, Cambridge, UK), phospho-SMAD2 (Ser465/467) (rabbit polyclonal, 1:1000, Cell Signaling Technology, Beverly, MA), SMAD2 (rabbit monoclonal, 1:1000, Cell Signaling Technology), TGF-β1 (sc-146; rabbit polyclonal, 1:1000, Santa Cruz Biotechnology, Inc., TX) and ACTB (anti β-actin, mouse monoclonal, 1:10 000, Sigma–Aldrich). After incubation with goat anti-rabbit or goat anti-mouse IgG horseradish peroxidase (HRP)-linked antibodies (1:10 000, Calbiochem, Merck Chemicals GmbH, Schwalbach, Germany), western blots were visualized with SuperSignal West PICO Chemiluminescent Substrate (Thermo Scientific, Rockford, IL). For Coomassie staining, SDS gels were incubated in staining solution [0.1% Brilliant Blue R 250 (Sigma–Aldrich) in 10% Methanol and 30% acetic acid] and destained in 10% Methanol and 30% acetic acid. Densitometry was performed with Total lab TL100 software (Nonlinear Dynamics, Durham, NC).
NaCl (TBS), was preincubated at 37, 42 or 46°C for 10 min before exposure to 120 μg/ml TPCK-trypsin (Sigma–Aldrich) at 37°C for 5 min. The reaction was stopped by adding 5× Laemmli Buffer and boiling the sample for 10 min at 90°C. Subsequently, the samples were subjected to western blot analysis with α-HTRA1 antibody.

Casein in vitro digestion

Bioactivity of HTRA1 protein from supernatant or purified HTRA1 was tested in vitro β-casein digestions. Twenty-micrograms of β-casein from bovine milk (Calbiochem) in 100 μl 50 mM Tris–HCl pH 7.6, 5 mM CaCl2 and 150 mM NaCl was mixed with 300 μl supernatant of transfected Hek293-Ebna cells or with purified HTRA1 protein (50 ng/μl in 100 μl 50 mM Tris–HCl pH 7.6, 5 mM CaCl2 and 150 mM NaCl). Samples were incubated at 37°C for a period of 3 h. After half an hour, 1, 2 and 3 h, 50 μl aliquots were taken and the reaction was stopped by adding 5× Laemmli Buffer and boiling the sample for 10 min at 90°C. Samples were resolved by SDS-PAGE and stained with Coomassie.

To compare β-Casein cleavage catalyzed by HTRA1:CG and HTRA1:TT, β-Casein was incubated with 300 μl serum-free medium of Hek293-Ebna cells transfected with expression constructs for HTRA1:CG and HTRA1:TT, respectively. The HTRA1 concentration was determined with Bio-Rad protein assay (Bio-Rad laboratories), adjusted to 15 ng/μl and controlled via western blot analysis with α-HTRA1 antibody.

TGF-β1 in vitro digestion

One microgram of recombinant TGF-β1 (PeproTech) in 100 μl 50 mM Tris–HCl pH 7.6, 5 mM CaCl2 and 150 mM NaCl was incubated with 300 μl serum-free medium of Hek293-Ebna cells transfected with the expression constructs for HTRA1:CG and HTRA1:TT, respectively. The HTRA1 concentration was determined by Bio-Rad protein assay (Bio-Rad laboratories), adjusted to 15 ng/μl and controlled via western blot analysis with α-HTRA1 antibody. Aliquots were collected at 0, 4, 8, 16 and 24 h and boiled with Laemmli Buffer to stop the reaction. Samples were subjected to western blot analysis with α-TGF-β1.

Reporter assay

MLEC-PAI/Luc cells were seeded into 96-well plates at a density of 1.5 × 10⁴ cells per well and treated with 2 ng/ml of recombinant TGF-β1 (PeproTech) in combination with 40 ng/ml of purified HTRA1 (Strep-tagged HTRA1:CG or HTRA1:TT) or eluate of empty vector. After 16 h, luciferase activity was determined as described previously (106).

Analysis of TGF-β signaling in BV-2 cells

BV-2 cells were seeded in 12-well cell culture plates at a density of 1.5 × 10⁵ (for immunocytochemistry) and in 6-well cell culture plates at a density of 5 × 10⁵ (for western blot analysis). To induce autocrine TGF-β signaling, cells were serum-starved for 2 h as described by Spitta and colleagues in 2015 (57) and then incubated with 40 ng/ml of Strep-tagged HTRA1 (HTRA1:CG or HTRA1:TT), or empty vector eluate for 2 h (SMAD signaling), or 3 and 24 h (PAI-1 expression).

Immunocytochemistry

Immunocytochemistry with α-phospho-SMAD2 (1:100, Cell Signaling Technology) and Cy3-conjugated rabbit secondary antibody (1:2000, Jackson ImmunoResearch, West Grove, NJ) was performed as described by Karlstetter and colleagues (106).

Quantitative real-time RT-PCR

Quantitative real-time RT-PCR was performed and analyzed as described (100). The following primers were used to detect transcripts of PAI-1 (mmPAI-1-FWD: 5’ AGA CAA TGG AGG GGC AAC AT 3’ and mmPAI-1-REV: 5’ TCT GAG GTC CAC TTT AGT CTC C 3’) and GNB2L (mmGNB2L-FWD: 5’ TCT GCA AGT ACA CGG TCC AG 3’ and mmGNB2L-REV: 5’ GAG AGC ATG ATA GGG TTG CTG 3’). PAI-1 expression was normalized to GNB2L.

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