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Polymorphism rs11200638 enhanced HtrA1 responsiveness and expression are associated with age-related macular degeneration

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OBJECTIVES: To investigate the role of polymorphism rs11200638 of high-temperature requirement factor A-1 (HtrA1) gene in the pathogenesis of age-related macular degeneration (AMD).
METHODS: Cultured adult retinal pigment epithelial cells (ARPE-19) expressing HtrA1 gene were treated with H_2O_2 or lipopolysaccharides (LPS) and analysed using western blot and quantitative polymerase chain reaction to illustrate the effects of oxidative and inflammatory stress on HtrA1 gene expression. Luciferase reporter plasmid driven by HtrA1 promoter with either normal allele G or risk allele A at SNP rs11200638 was transfected to ARPE-19 cells to investigate the effect of the G/A variation on HtrA1 promoter activity. The effects of HtrA1 overexpression on ARPE-19 cells were analysed with respect to percentage of cell proliferation inhibition and cell apoptosis.
RESULTS: HtrA1 expression was significantly increased with LPS or H_2O_2 stimulations ($p < 0.05$). In ARPE-19 cells, HtrA1 promoters (-1 to -2175 bp from translation starting point) with risk allele A or normal G at rs11200638 did not show statistically significant differences in their luciferase reporter expression ($p = 0.054425173$), however, both promoters showed a persistent trend of higher luciferase expressions after 100 ng/ml LPS treatment. The luciferase expression level was significantly greater in the promoter with risk A when compared to that with normal G. Overexpression of HtrA1 resulted in apoptosis of ARPE-19 cells with $53.8 \pm 1.6\%$ of proliferation inhibition ($p < 0.01$).
CONCLUSIONS: Risk haplotype A at rs11200638 significantly increased the responsiveness of HtrA1 promoter to inflammation and subsequently enhanced HtrA1 expression. HtrA1 overexpression induced ARPE-19 apoptosis and growth inhibition, relevant to pathogenesis of AMD.

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INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of blindness in elder individuals, accounting for 8.7% of blindness globally [1]. Both environmental and genetic risk factors are involved in AMD pathogenesis [2, 3]. Major progress has been made in determining the genetic basis of AMD through the identification of susceptibility gene loci [4–6]. Among those reported loci, high-temperature requirement factor A-1 (HtrA1) gene has been shown to be one of the major causal genes to AMD [7–9]. Certain single nucleotide polymorphisms (SNPs) including rs11200638, rs2672598, rs1049331 and rs2293870 in the promoter region and the first exon of HtrA1 gene have been identified to be implicated in AMD [10–12].

SNP rs11200638 in HtrA1 promoter site was deemed as the AMD-associated allele primarily based on the assumption that rs11200638 upregulated HtrA1 expression. While previous studies suggested that variant rs11200638 increased the expression of

HtrA1 and the susceptibility to AMD [13–15]. Yang et al. reported that rs11200638 with risk allele was correlated with elevated HtrA1 expression in lymphocytes and RPE tissue from AMD patients [14]. Subsequently, this correlation was confirmed in the retinas of patients with AMD [13, 16–19]. However, the potential role of HtrA1 encompassed by the rs11200638 haplotype in the pathogenesis of AMD remains unclear, because the previous evidences pertaining to the correlation between rs11200638 genotypes and HtrA1 expression levels were conflicting [20–23]. Some reporters claimed that variant rs11200638 increased the expression of HtrA1, but others claimed that the genotypes at rs11200638 were not correlated with HtrA1 expression. We speculated that micro-environmental risk factors were necessary to determine the role of AMD-associated allele on the pathogenesis of AMD. Variant rs11200638 may alter the HtrA1 promoter responsiveness to those risk factors, i.e., oxidative stress and inflammation, relevant to the pathogenesis of AMD.

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Retinal pigment epithelium (RPE) senescence induced by oxidative stress is one of the key contributors to early AMD and drusen formation [11]. The role of inflammation in early stage has been implicated in the pathogenesis of late-stage AMD [24]. RPE cell, one of the most important cells involved in the pathogenesis of AMD, is essential for maintaining vision by keeping photoreceptors healthy [25]. There is a mutualistic relationship between the components of the photoreceptor/RPE/Bruch's membrane/choriocapillaris complex. Aging and the accumulated effects of environmental stresses may result in death and dysfunction of all of the components in the complex and progress to AMD. HtrA1 expression and its promoter activity were investigated in cultured adult RPE (ARPE-19) cells. The effects of overexpression of HtrA1 on ARPE-19 cell growth were also studied.

MATERIALS AND METHODS

Cell culture
ARPE-19 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM, low glucose formulation, 11330-033; Gibco, Invitrogen, CA, USA) supplemented with 10% (v/v) foetal bovine serum (FBS, 04-001-1ACS; BI, US origin), 100 U/ml penicillin and 100 μg/ml streptomycin (10378-016; Gibco).

STR (short tandom repeat) analysis
The STR analysis was performed on the cell line to genotype the cell line by iCell Bioscience Inc (Shanghai, China). DNA was extracted using the Axygen genomic DNA extraction kit (200600; Corning, USA), and the STR loci and gender gene Amelogenin were detected using the ABI 3730XL genetic analyser according to the 20-STR amplification protocol.

LPS (lipopolysaccharides) and H$_2$O$_2$ stimulation of ARPE-19 cells
ARPE-19 cells grown to 70–80% cell confluence in six-well cell plates were incubated overnight in fresh DMEM without FBS, and subsequently treated with various concentrations of lipopolysaccharides (LPS, 01062003; DIFCO, Livonia, MI, USA) or H$_2$O$_2$. For inducing oxidative stress, H$_2$O$_2$-conditioned media was used at final concentrations of 0.2, 2, 20 or 200 μM H$_2$O$_2$, respectively, in DMEM for 24 h, by dissolving 30% H$_2$O$_2$ stock solution (Q18755; Fisher Scientific, Fair lawn, NJ, USA) before use. For inducing inflammation, LPS was used at final concentrations of 0.1, 1, 10 or 100 ng/ml, respectively, in DMEM for 24 h. The treated cells were investigated for HtrA1 protein and mRNA expression level using western blot and quantitative polymerase chain reaction (qPCR), respectively. The doses of LPS and H$_2$O$_2$ that increased HtrA1 expression significantly were defined as effective doses.

Western blot analysis
The ARPE-19 cells were lysed in lysis buffer containing 1% Triton X-100 (0694; Amersco, OH, USA), 1% deoxycholate (0613; Amersco), and proteinase inhibitor cocktail (04693132001; Roche, Rotkreuz, Switzerland). Total protein concentrations were measured using a modified Lowry protein assay kit (500-0001; BioRad, CA, USA). Equal amounts of denatured cellular protein (25 μg) from each sample were applied to 10% SDS-polyacrylamide gels, and resolved proteins were transferred to nitrocellulose membrane for sequential probing with specific antibodies, i.e., rabbit polyclonal anti-htrA1 antibody (ab38610; Abcam, MA, USA), anti-superoxide dismutase 1 (SOD1) antibody (2582-1; Epitomics, CA, USA) or monoclonal antibody against interleukin-6 (IL-6) (1457-1; Epitomics, CA, USA), respectively, and the secondary antibody against rabbit IgG conjugated with horseradish peroxidase (611035215; Jackson ImmunoResearch, PA, USA). PBS-treated cells were used as negative control. In order to avoid the false-positive caused by non-specific adsorption, the sample without cells was used as a negative control in the experiment. The blots were developed with BeyoECL plus enhanced chemiluminescence detection kit (P0018S; Beyotime, Shanghai, China). Band density was analysed using Quantity One software (Bio-Rad, CA, USA) and the grey value (area under the optical density distribution curve) was used to determine the relative protein levels.

Preparation of RNA
Total RNA was isolated from the cultured ARPE-19 cells with TRIzol Reagent (15596026, Invitrogen, CA, USA) following the manufacturer's instructions. The RNA pellets were washed with 75% ethanol and dissolved in RNase-free water. The residual DNA was digested with DNase I. Concentration and purity (i.e., OD260/OD280) of RNA samples were monitored with a SmartSpec Plus spectrophotometer (Bio-Rad, CA, USA). RNA integrity was evaluated by examining the 28S and 18S rRNA bands after separation in a 1% agarose gel.

QPCR analysis
Total RNA was reverse-transcribed to complementary DNA (cDNA) using RevertAid™ first-strand cDNA synthesis kit (K1622, Fermentas, Vilnius, Lithuania). In brief, a 12 μl mixture containing 1 μg RNA and 1 μl oligo (dT) 18 (0.5 μg/μl) was incubated at 65 °C for 5 min, followed by adding 4 μl reaction buffer, 1 μl RNase inhibitor (20U/μl), 2 μl dNTP Mix (10 mM), and 1μl M-MuLv Reverse Transcriptase (200 U/μl). Then, the total reverse-transcription system above was incubated sequentially at 42 °C for 60 min and 70 °C for 5 min.

QPCR was carried out on iCycler-iQ5® (Bio-Rad, CA, USA) as described [26]. In brief, a 20 μl reaction mixture, containing 1× SoFast EVAGreen supermix (1725202, Bio-Rad), 2 μl of cDNA, and 330 nM of each of the forward and reverse primers, was cycled sequentially, 30 s at 95 °C for one cycle, 5 s at 95 °C and 10 s at optimum annealing temperature (Table 1 of Supplementary materials) for 40 cycles. Primers were synthesised (HPLC purification grade) at Beijing Genomics Institute, China (Supplementary Table 1). Assays for each cDNA sample were performed in triplicate (maximum ΔCt of replicate samples <0.5). Relative expression calculations, corrected with PCR efficiency and normalised versus the reference gene GAPDH (NM002046.3; GenBank), were performed with IQ 5 software (Bio-Rad). The results were expressed as the ratio of specific mRNA/GAPDH mRNA. The experiment was repeated three times for consistency.

Luciferase reporter assays
A DNA fragment containing −1 to −2175 bp from the HtrA1 translation site including protective haplotype G allele at rs11200638 (wild type, WT) was amplified from genomic DNA of an individual using nested-PCR with the following primers: inner forward, CGACGCGTCGCTGGAGGGGAGGAGG GGT, inner reverse, CCCAAGCTTGGCGACTCTGGCGGCGGC; outer forward, GGGCACGAGGATGGAAGAGG, outer reverse, AGCAGCAGCGGGAGAA-GAGC. The construct was subcloned into the Mlu I- Hind III site of the pGL3-Basic vector (E1751, Promega, WI, USA), and then verified using complete bidirectional DNA sequencing. The risk haplotype construct carrying risk A allele at rs11200638 (mutant type, MT) was generated using site-directed mutagenesis kit (210518, Agilent Technologies, CA, USA), with the following primers: forward, GGACGCTGCCTTCGTCCAGCCGCAGAGGCC CCG, and reverse, GGGGCCTCTGCGGCTGGACGAAGGCAGCGTCCGC. The pGL3-Basic vector without insert (negative) was transfected into ARPE-19 cells as a negative control. A positive control plasmid (pGL3-control Vector, E1741, Promega, WI, USA) containing an SV40 enhancer and promoter driving luciferase reporter was obtained from Promega.

ARPE-19 cells were split into 96-well plates and cultured overnight, then co-transfected with 1 ng of the transfection control Renilla luciferase plasmid pTK-RL (Promega) and 200 ng of WT or MT plasmid. Transfections were performed using the Lipofectamine® LTX&PLUS™ Reagent (15338100; Invitrogen, CA, USA). Each type of plasmid was transfected in three wells simultaneously. Twenty-four hours after transfection, cells were washed twice with PBS and then harvested. Luciferase activities were measured using the Luciferase Assay Kit (E1910; Promega, WI, USA). Fold induction was derived relatively to the normalised reporter activity.

To investigate the effect of inflammatory and oxidative stress on the HtrA1 reporter activity, after pre-treatment with 200 μM H$_2$O$_2$ or 100 ng/ml LPS for 6 h to induce cellular stress, ARPE-19 cells were then transiently transfected with a luciferase reporter plasmid driven by the HtrA1 promoter, with WT or MT at SNP rs11200638. Six hours after transfection, ARPE-19 cells were further treated with 200 μM H$_2$O$_2$ or 100 ng/ml LPS for 24 h. Luciferase activities were finally measured as described above.

Cloning of human HtrA1
A 1443-bp open reading frame of the HtrA1 gene (NM 002775.4; GenBank) was cloned into an empty OmicsLink™ expression clone pReceiver-M06 (GeneCopoeia, Guangzhou, China) to obtain HtrA1 expression clone of EX-M0558-M06. The information pertaining to the vector and restriction
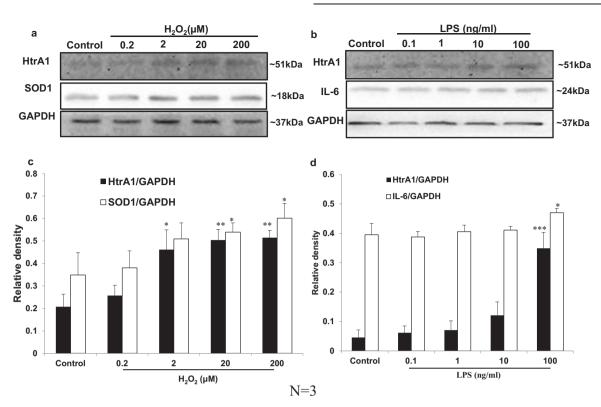
Fig. 1   Western blot analysis of HtrA1 expression in ARPE-19 cells treated with H_2O_2 or LPS. Serum-starved ARPE-19 cells were plated on dishes in medium supplemented with varying concentrations of H_2O_2 (**a**) or LPS (**b**) for 24 h and then lysed. The lysates were analysed by western blot. The control was performed with vehicle. Blots were developed with HRP-conjugated secondary antibodies and an enhanced chemiluminescence detection kit. **c**, **d** Densitometry of three independent experiments. The western blot gels were cropped according to the molecular size of protein. Band density (grey value) was analysed using Quantity One software and used to determine the relative protein level. Results were expressed as means ± SD ($n = 3$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with control.

enzyme for EX-M0558-M06 was presented in Fig. 4a. The construct was verified by direct sequencing. Sub-confluent ARPE-19 cells in 96-well plate were transfected with 3 µg constructed plasmid per well in transfection reagent (Lipofectamine® LTX&PLUS™ Reagent). Twenty-four hours after transfection, expression of the recombinant human HtrA1 protein was validated by western blot.

Cell proliferation assay
Cell proliferation was analysed using Cell Counting Assay Kit-8 (CCK-8) (DJDB4000X, Dojindo Molecular Technologies, Kumamoto, Japan). Briefly, 100 µl of ARPE-19 cell suspension (5×10^3 cells/well) was dispensed into a 96-well plate and pre-incubated overnight, then transfected with 3 µg HtrA1 construct EX-M0558-M06 per well in transfection reagent (Lipofectamine® LTX&PLUS™ Reagent). Twenty-four hours after transfection, 10 µl CCK-8 solutions were added to each well. After an additional incubation for 1 h, the absorbance at 450 nm was measured using a microplate reader. The inhibition percentage of cell proliferation was recorded using transfection reagent treated cells at 100%.

Cell apoptosis detection
Cell apoptosis was analysed using the Annexin V-FITC/propidium iodide (PI) apoptosis assay kit (KGA108; KeyGEN Biotech, Nanjing, China). Briefly, 24 h after transfection with HtrA1 construct EX-M0558-M06, ARPE-19 cells were harvested and washed twice with cold PBS (pH 7.4) and stained with 3 µl of Annexin V and 3 µl of PI in 300 µl of binding buffer for 10 min in the dark. The number of apoptotic cells was analysed using Becton Dickinson FACScan Flow Cytometer (Becton Dickinson, MD, USA).

Statistical analysis
Each sample was triplicated. Every experiment was repeated at least three times for consistency. The experimental data were expressed as means ($\bar{x}$) ± standard deviation (SD), and analysed with software SPSS Statistics 17.0 (SPSS Inc., IL, USA) for group-wise comparisons and statistical analyses.

T-test was used for comparison between two groups. Differences were considered statistically significant when $P < 0.05$.
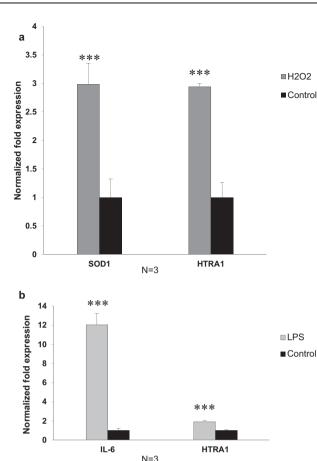
RESULTS
STR analysis
The DNA genotype of the cell line was found to completely match in the cell line retrieval, which showed that the cells named ARPE-19 and ARPE-19/HPV-16 in DSMZ database. The cell numbers were related to CRL-2302 and CRL-2502, respectively. No multiple alleles were found in this cell line. The results of the genotype were listed in Supplementary materials (including Supplementary Table 2).

H_2O_2-mediated induction of HtrA1 expression in ARPE-19 cells
To investigate the involvement of oxidative stress in HtrA1 expression, ARPE-19 cells were treated with diverse concentrations of H_2O_2 for 24 h. The effects of oxidative stress were evidenced by the increase in SOD-1 protein level (Fig. 1a, c). Western blot result showed that H_2O_2 at concentrations of 2, 20 and 200 µM induced 2.23 ± 0.42 ($n = 3$, $p < 0.05$), 2.43 ± 0.23 ($n = 3$, $p < 0.01$), and 2.48 ± 0.15-fold ($n = 3$, $p < 0.01$) increases in HtrA1 protein level, respectively (Fig. 1a, c).

Similarly, qPCR data showed a 2.94 ± 0.06-fold ($n = 3$, $p < 0.001$) significant increase in HtrA1 mRNA expression level with the minimum effective dose of 200 µM H_2O_2 (Fig. 2a). These results suggest that oxidative stress simulated by H_2O_2 may enhance HtrA1 expression at both the mRNA and protein levels. Due to the volatility of H_2O_2, the maximum effective dose of 200 µM H_2O_2 was used in subsequent experiments to obtain relative certain oxidative stress induction for evaluation of the HtrA1 promoter activity.

a

b

Fig. 2 Changes in the relative expression of HtrA1 mRNA expression in ARPE-19 cells treated with H_2O_2 or LPS. Serum-starved ARPE-19 cells were plated on dishes in medium supplemented with $2\,\mu M$ H_2O_2 (**a**) or 100 ng/ml LPS (**b**) for 24 h. The control was performed with vehicle. Total RNA extracted from those ARPE-19 cells was subjected to qPCR analysis and normalised with that of the reference gene GAPDH as described in "Methods". Results were expressed as means $\pm$ SD ($n = 3$). ***$p < 0.001$ vs. control.

LPS-mediated induction of HtrA1 expression in ARPE-19 cells
To determine the effects of inflammation on HtrA1 expression, ARPE-19 cells were stimulated with different concentrations of LPS for 24 h. The effects of inflammation were evidenced by the increase in IL-6 protein level (Fig. 1b, d). Western blot results showed that HtrA1 protein expression increased at doses of 1, 10 and 100 ng/ml and induced a 1.55 ± 0.69 ($n = 3$, $p > 0.05$), 2.65 ± 1.00 ($n = 3$, $p > 0.05$), and 7.66 ± 1.18-fold ($n = 3$, $p < 0.001$) increases in HtrA1 protein level, respectively (Fig. 1b, d). HtrA1 protein expression after using the maximum dose of 100 ng/ml of LPS was statistically significantly increased when compared to the control (Fig. 1d). Hence, the effective dose of 100 ng/ml LPS was used in subsequent experiments to induce inflammatory stress for evaluation of the HtrA1 promoter activity.

Consistent with the results obtained from the western blot, qPCR data showed a 1.92 ± 0.11-fold ($n = 3$, $p < 0.001$) increase in HtrA1 mRNA expression (Fig. 2b) with the effective dose of 100 ng/ml LPS induction. These findings suggest that inflammation stress stimulated by LPS may induce HtrA1 over-expression at both the mRNA and protein levels.

Effect of rs11200638 on HtrA1 expression
The SNP rs11200638, located in the promoter region of HtrA1 gene, is highly associated with development of AMD. To evaluate the effects of SNP rs11200638 on HtrA1 promoter activity, as in
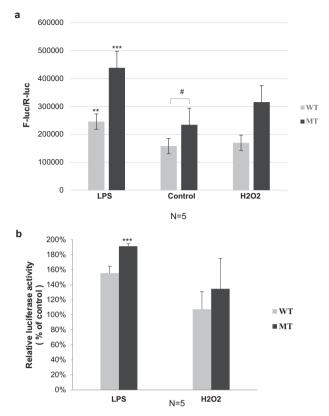
a

b

Fig. 3 Luciferase activities in cultured ARPE-19 cells transfected with HtrA1 promoter reporter constructs after treated with LPS or H_2O_2. Effects of the rs11200638 variants on HtrA1 expression in ARPE-19 cells. After pre-treatment with 100 ng/ml LPS or $200\,\mu M$ H_2O_2 for 6 h to induce cellular stress, ARPE-19 cells were transiently transfected with WT or MT HtrA1 promoter, another 6 h after transfection, ARPE-19 cells were further treated with 100 ng/ml LPS or $200\,\mu M$ H_2O_2 for 24 h. The control was performed with vehicle. Normalised luciferase activity was measured in five independent experiments and shown in (**a**). Results were expressed as means $\pm$ SD. **$p < 0.01$ vs. WT control treated with LPS; ***$p < 0.001$ vs. MT control treated with LPS. #$P = 0.054425173$. The relative luciferase activity (% of control) was shown in (**b**). ***$p < 0.001$ vs. LPS-treated control.

previous reports [13, 14], ARPE-19 cells were transiently transfected with a luciferase reporter plasmid driven by the HtrA1 promoter, carrying either allele G (WT) or the risk allele A (MT) genotype at SNP rs11200638. In ARPE-19 cells, WT HtrA1 promoter and MT did not show statistically significant differences in their luciferase reporter expression ($P = 0.054425173$, Fig. 3a).

Effect of inflammatory and oxidative stress on HtrA1 expression
To evaluate the effect of inflammatory and oxidative stress on the promoter activity, ARPE-19 cells were transfected with either WT or MT reporter plasmid encompassed with SNP rs11200638, then further treated with identified most effective dose of $200\,\mu M$ H_2O_2 or 100 ng/ml LPS, luciferase activities were then measured as described above. Both MT and WT showed a persistent trend of higher relative luciferase expressions compared with their respective control when treated with LPS or H_2O_2 ($P < 0.001$ and $P < 0.01$, respectively) (Fig. 3a). When treated with 100 ng/ml LPS, the fold increase of luciferase expression in MT group was significantly greater than that in the WT ($P < 0.001$) (Fig. 3b). These results suggest that the risk haplotype (AA) of SNP rs11200638 increased the responsiveness of HtrA1 promoter to inflammation induction.
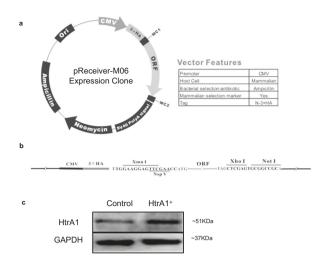
Fig. 4 Overexpression of HtrA1 in cultured ARPE-19 cells. a, b Vector and restriction enzyme information for EX-M0558-M06. **c** Western blot analysis of HtrA1 expression in ARPE-19 cells after transfected with the expression vector hosting HtrA1 cDNA. HtrA1$^+$, ARPE-19 cells transfected with HtrA1 construct EX-M0558-M06; Control, empty vector control.

Effects of HtrA1 overexpression on the growth of ARPE-19 cells

RPE cell is a crucial component in the pathogenesis of AMD [25, 27]. Both local inflammation and oxidative stress are related to RPE apoptosis in AMD [12, 14, 28]. To investigate the effects of HtrA1 overexpression on the growth of RPE, ARPE-19 cells were transfected with an expression vector containing HtrA1 cDNA (Fig. 4a, b). Western blot analysis revealed an overexpression of HtrA1 in the transfected ARPE-19 cells (Fig. 4c). Overexpression of HtrA1 resulted in significant apoptotic changes in ARPE-19 cells and $53.8 \pm 1.6\%$ ($n = 5$) of proliferation inhibition in ARPE-19 cells using CCK-8 assay ($P < 0.01$) (Fig. 5a, b).

To elucidate whether the growth-inhibitory effect was related to the induction of apoptosis, flow cytometry analysis was performed on the Annexin V-FITC/PI stained ARPE-19 cells. It was determined that the overexpression of HtrA1 induced apoptotic process in ARPE-19 cells, with a higher percentage of early apoptotic cells in the transfected cells compared to the control (Fig. 5c, 45.2% vs 1.1%).

DISCUSSION

Various aetiological factors including advanced age, micro-environmental risk factors and inherited susceptibility gene(s) have been identified to be involved in the pathogenesis of AMD. Oxidative stress, an important micro-environmental risk factor, results in significant structural and functional changes in RPE, which could result in RPE senescence and drusen formation in early AMD [11, 24, 29]. The previous study suggested that the oxidative damage and immune response contributing to AMD [30]. Kauppinen et al. also demonstrated that oxidative stress can activate NLRP3 inflammasomes in RPE cells which occupy centre stage in the pathogenesis of AMD [31].

HtrA1 protein is involved in many diseases, including AMD. However, the mechanisms underlying regulation of HtrA1 expression remain to be determined. Supanji et al. suggested that oxidative stress induced by H_2O_2 increased HtrA1 expression during premature cell senescence [32]. Hou et al. have demonstrated that toll-like receptor-4 (TLR4) activation by LPS significantly induced HtrA1 expression in fibroblasts and macrophages [33]. In our study, diverse doses of H_2O_2 and LPS were independently applied in cultured ARPE-19 cells to mimic

oxidative stress and inflammation, which were evidenced by the significant increases in SOD-1 and IL-6 protein level, respectively ($p < 0.05$). The HtrA1 expressions at both protein and mRNA increased with the identified concentrations of H_2O_2 and LPS inductions. The concomitant augmentation of SOD-1 and IL-6 with HtrA1 expression suggests a potential modelling of HtrA1 in the pathogenesis of AMD at a molecular basis under the oxidative stress and inflammation. However, results from other independent groups showed that the genotypes at rs11200638 were not correlated with HtrA1 expression at either mRNA or protein level either in the retina and white blood cells [21, 23]. Wang et al. reported that rs11200638 genotype has no effects on HtrA1 expression after examining 24 retina-RPE-choroids samples [21]. Furthermore, they studied AMD-associated variants at chromosome 10q26 locus and the expression of HtrA1 in 82 retina-RPE-choroids samples and suggested that there was no significant change of HtrA1 mRNA level [34]. In the current study, we also demonstrated that upregulation of HtrA1 expressions induced by H_2O_2 and LPS were in a dose-dependent manner. HtrA1 had been identified as one of the most important genes for the risk of advanced AMD [35]. Previous studies suggested that overexpression of HtrA1 may alter the integrity of Bruch's membrane, favour neovascularization, and promote the invasion of choroid capillaries across the extracellular matrix, as what happened in wet AMD or advanced AMD [22, 36], suggesting that a certain threshold of oxidative and inflammative damage may be required to induce significant changes in HtrA1 expression. We speculated that the upregulation process on HtrA1 gene expression in vivo may not occur until the late stage of the disease, and therefore may explain the above incongruous results on HtrA1 expression.

AMD-associated variant rs11200638 locates in HtrA1 promoter site and influences the transcriptional regulation of HtrA1 gene [14]. With the hypothesis that the variant rs11200638 alters the responsiveness of HtrA1 promoter and subsequently enhances the HtrA1 expression under oxidative stress and inflammation, the luciferase reporter plasmids driven by HtrA1 short promoter (−1 to −2175 bp from translation site), encompassed with either protective allele G (WT) or risk allele A (MT) at rs11200638, were transfected into ARPE-19 cells. The identified maximum effective doses of H_2O_2 and LPS were used to induce AMD-like oxidative and inflammative damage in cells. Before the introduction of H_2O_2 or LPS, we measured the luciferase expression and found that there were no statistically significant differences in luciferase reporter either with WT or MT genotype. After ARPE-19 cells were treated with LPS, reporter plasmid with both MT and WT HtrA1 promoter exhibited higher luciferase expressions compared with their respective controls. And samples with MT exhibited a significant higher fold increase in luciferase expression when compared to that with WT samples ($p < 0.001$), indicating that inflammatory damage involved in variant rs11200638 upregulating HtrA1 promoter responsiveness. Risk haplotype A had a more significant effect on altering HtrA1 promoter activity, as demonstrated by a greater fold increase in luciferase expression in MT group when compared to that in WT group.

Variant rs11200638 locates in a conserved CpG island, which extended 2.1 kb upstream from the transcription starting site within promoter region of HtrA1 gene. AMD-risk allele A may disrupt the CpG island [14] by removing the inhibition effect on methylated CpG island, increasing the responsiveness of HtrA1 gene to the micro-environmental factors. However, the mechanism of over-expression of HtrA1 gene in RPE cells remains unclear and requires investigation, it has been described that LPS can induce HtrA1 expression in human synovial fibroblasts and monocyte/macrophages via the classical NF-κB pathway [33]. And, TLR4 activation from LPS results in preferential binding of NF-κB p65 to the HtrA1 promoter, which subsequently induces the upregulation of HtrA1 transcription [33].
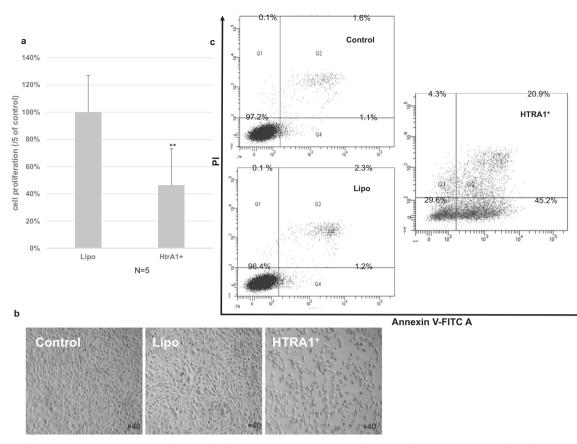
Fig. 5 Effects of HtrA1 overexpression on the growth of ARPE-19 cells. a Cell proliferation of ARPE-19 cells transfected with HtrA1 construct EX-M0558-M06 analysed using CCK-8, and the result was recorded using lipo-treated cells at 100%. **b** Cell morphology of ARPE-19 cells after transfected with the HtrA1 construct EX-M0558-M06. The magnification is ×40. **c** Flow cytometric evaluation of ARPE-19 apoptosis. Histograms derived from flow cytometry comparing apoptotic cells between vehicle and HtrA1 transfected cells. Twenty-four hours after transfection, the induction of apoptosis was determined using flow cytometric analysis of Annexin V-FITC and PI-stained ARPE-19 cells. Cells in the lower right quadrant indicate Annexin positive, early apoptotic cells, and cells in the upper right quadrant indicate Annexin positive/PI positive, late apoptotic cells. Results were expressed as means ± SD. **$p < 0.01$ vs. Lipo-treated control. Control, non-transfected ARPE-19 cells. Lipo, performed with Lipofectamine® LTX&PLUS™ reagent. HtrA1+, transfected with HtrA1 construct EX-M0558-M06.

We noticed that HtrA1 expression and its promoter potency did not respond similarly to the oxidative stress induction. That is, certain doses of H_2O_2 stimulations significantly increased the HtrA1 expressions at protein and mRNA level($p < 0.05$), but did not present corresponding effects on its promoter potency, neither with MT nor MT ($p > 0.05$). We speculated that the short promoter (-1 to -2175bp from translation starting point) constructs in the study did not harbour those necessary response elements for transcription factor(s) elicited by oxidative stress; or that the AMD risk, conferred by this region is potentially driven by multiple variants, such as LOC387715 insertion/deletion(ins/del) and rs2672598, which were not encompassed or investigated with our promoter constructs [18, 37].

In our study, we only focused on the role of single variant rs11200638 in the promoter region of HtrA1 gene. Previous studies [18, 21, 23, 38] suggested AMD risk conferred by HtrA1 gene may be driven by multiple alleles in risk haplotype with binary model. The intergenic region between LOC387715 (ins/del) and the SNP rs11200638, rs3793917, and rs11200638 in the HtrA1 gene were all reported the likely sites to be significantly associated with AMD [18, 39]. Further studies may combine other AMD-associated variants at chromosome 10q26 region to refine our understanding of their role on HtrA1 gene.

HtrA1 gene is thought to control extra-cellular matrix degradation and RPE cell growth or survival [11, 40–42] and enhance cell senescence through p38 MAPK pathway [32]. Excessive HtrA1

accumulation results in drusen formation which may compromise Bruch's membrane integrity, favouring the invasion of choroid capillaries across the extra-cellular matrix, as occurs in wet AMD [11, 14]. We found that the overexpression of HtrA1 significantly inhibited the growth of ARPE-19 cells. Apoptotic processes were involved in the growth inhibition from enhanced HtrA1 expression on RPE cells. This functional property of HtrA1 could explain the enhanced expression of this protein in dry and wet AMD lesions, both preceded by early and intermediate stages, which are characterised by RPE pigmentary abnormalities and drusen. The primary clinical characteristic of advanced dry AMD is the appearance of RPE cell atrophy, which is the consequence of stressed RPE dying and defects. The loss of RPE could lead to the gradual degeneration of nearby photoreceptors and progressive visual impairment [43]. We also demonstrated that HtrA1 protein increased significantly in cultured ARPE-19 cell nuclei when it was treated with the effective dose of 100 ng/ml LPS or 200 µM H_2O_2 (data not shown). Whether the accumulation of HtrA1 protein in nuclei increased its role in AMD pathogenesis remains unclear, further studies are needed to address it.

In our study, diverse doses of H_2O_2 and LPS were tested for the induction of oxidative and inflammatory reactions. The identified maximum effective doses of H_2O_2 and LPS were finally applied to treat ARPE-19 cells to imitate the AMD-like damage. We found variant rs11200638 upregulated the responsiveness of HtrA1 promoter after inflammation induction. However, the involvement

of AMD-risk factors to AMD pathogenesis may be more complex in vivo.

So, it suggests that oxidative stress and inflammation increased HtrA1 expression. Variant rs11200638 enhances the responsiveness of HtrA1 promoter with LPS induction. The increased HtrA1 expression induces RPE cell apoptosis and growth inhibition.

SUMMARY

What was known before

- Both environmental and genetic risk factors are involved in AMD pathogenesis.
- High-temperature requirement factor A-1 (HtrA1) gene has been shown to be one of the major causal genes to AMD.
- SNP rs11200638 in HtrA1 promoter site was deemed as the AMD-associated allele primarily based on the assumption that SNP rs11200638 up-regulated HtrA1 expression.

What this study adds

- In the present study, diverse doses of H_2O_2 and LPS were tested for the induction of oxidative and inflammatory reactions.
- We found variant rs11200638 upregulated the responsiveness of HtrA1 promoter after inflammative induction.
- The increased HtrA1 expression induces RPE cell apoptosis and growth inhibition. However, the involvement of AMD-risk factors to AMD pathogenesis may be more complex in vivo eyes.

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AUTHOR CONTRIBUTIONS

FH, XL, SC, and XL conceived and designed the presented study. FH, XL, SC, LL, TZ and MY performed the data collection. FH, XL, SC, and XL performed the analysis and wrote the manuscript. XW and NF provided critical review of the manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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