A genome-wide association study identified a novel genetic loci
STON1-GTF2A1L/LHCGR/FSHR
for bilaterality of neovascular age-related macular degeneration

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Bilateral neovascular age-related macular degeneration (AMD) causes much more handicaps for patients than unilateral neovascular AMD. Although several AMD-susceptibility genes have been evaluated for their associations to bilaterality, genome-wide association study (GWAS) on bilaterality has been rarely reported. In the present study, we performed GWAS using neovascular AMD cases in East Asian. The discovery stage compared 581,252 single nucleotide polymorphisms (SNPs) between 803 unilateral and 321 bilateral Japanese cases but no SNP showed genome-wide significance, while SNPs at six regions showed P-value < 1.0 × 10−5, STON1-GTF2A1L/LHCGR/FSHR, PLXNA1, CTNNA3, ARMS2/HTRA1, LHFP, and FLJ38725. The first replication study for these six regions comparing 36 bilateral and 132 unilateral Japanese cases confirmed significant associations of rs4482537 (STON1-GTF2A1L/LHCGR/FSHR), rs2284665 (ARMS2/HTRA1), and rs8002574 (LHFP) to bilaterality. In the second replication study comparing 24 bilateral and 78 unilateral cases from Singapore, rs4482537 (STON1-GTF2A1L/LHCGR/FSHR) only showed significant association. Meta-analysis of discovery and replication studies confirmed genome-wide level significant association (P = 2.61 × 10−9) of rs4482537 (STON1-GTF2A1L/LHCGR/FSHR) and strong associations (P = 5.76 × 10−7 and 9.73 × 10−7, respectively) of rs2284665 (ARMS2/HTRA1) and rs8002574 (LHFP). Our GWAS for neovascular AMD bilaterality found new genetic loci STON1-GTF2A1L/LHCGR/FSHR and confirmed the previously reported association of ARMS2/HTRA1.

Age-related macular degeneration (AMD) is one of the major causes of visual impairment in developed countries. Although early stage AMD does not affect visual function, late stage AMD induces severe visual loss. AMD is a

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complex disease caused by multiple environmental and genetic risk factors. Previous genome-wide association studies (GWASs) identified two major susceptibility loci for AMD; complement factor H (CFH) and age-related maculopathy susceptibility 2/high temperature requirement A1 (ARMS2/HTRA1). Recently, AMD Gene Consortium performed meta-analysis of GWASs and found 34 loci were associated with AMD development. Furthermore, GWASs in East Asian populations revealed new loci for AMD and suggested ethnic differences in susceptibility to AMD.

Compared with patients with unilateral late AMD, patients with bilateral late AMD are more prone to visual handicaps. The prevalence of bilateral AMD was reported to be 40–50% in Caucasian and 10–20% in Asian. Previous studies investigated the association between bilaterality of AMD and the known AMD susceptibility loci. Several studies reported that ARMS2/HTRA1 contributes to the bilaterality of late AMD. In contrast, it is still controversial whether CFH increases the risk of AMD bilaterality. To identify the genetic determinants associated with bilaterality of late AMD in East Asian, we conducted a GWAS comparing bilateral late AMD patients with unilateral late AMD patients. Since most late AMD is neovascular AMD (wet type) in East Asian and geographic atrophy (dry type) is rare, we focused on only neovascular AMD. After finding genes associated with the bilaterality of neovascular AMD, we confirmed their susceptibility to AMD occurrence by comparing all neovascular AMD cases including both bilateral cases and unilateral cases with controls of Japanese general populations.

Results
Japanese patients with neovascular AMD were recruited at the Center for Macular Diseases of Kyoto University Hospital (n = 821) and Fukushima Medical University (n = 333) for the discovery stage. From these 1154 cases, 10 cases were excluded due to lack of detailed fundus examination of the fellow eye and 20 cases were excluded because of the quality control for their genotype count analysis. Of the 1124 cases analyzed in the discovery stage, 803 had unilateral neovascular AMD and 321 had bilateral neovascular AMD. Subtypes of AMD were

| Discovery stage | Bilateral neovascular AMD | Unilateral neovascular AMD | P-value |
|-----------------|--------------------------|---------------------------|---------|
|                 | n | Age | Sex (%) | Age | Sex | Age | Sex |
| Kyoto           | 247 | 80.9 ± 7.8 | 70.5 | 546 | 76.4 ± 8.4 | 69.6 | <0.001 | 0.81 |
| Fukushima       | 74 | 81.6 ± 6.3 | 83.8 | 257 | 77.6 ± 7.9 | 75.1 | <0.001 | 0.12 |
| Total           | 321 | 81.1 ± 7.4 | 73.5 | 803 | 76.8 ± 8.3 | 71.4 | <0.001 | 0.47 |
| Replication stage | 10 cases were excluded due to lack of detailed fundus examination of the fellow eye and 20 cases were excluded because of the quality control for their genotype count analysis. Of the 1124 cases analyzed in the discovery stage, 803 had unilateral neovascular AMD and 321 had bilateral neovascular AMD. Subtypes of AMD were

| Replication stage | n | Age | Sex (%) | Age | Sex | Age | Sex |
|-------------------|---|----|--------|----|-----|----|
| Kobe              | 36 | 83.4 ± 7.0 | 77.8 | 132 | 77.4 ± 8.0 | 71.2 | <0.001 | 0.43 |
| Singapore         | 24 | 71.6 ± 5.9 | 70.8 | 78 | 66.0 ± 10.2 | 61.5 | 0.0013 | 0.56 |

Table 1. Neovascular age-related macular degeneration samples used in the study.

Figure 1. Minus log-transformed P-values are shown in a signal intensity (Manhattan) plot relative to their genomic position for bilaterality of age-related macular degeneration. P-values are adjusted for age and sex.
| SNP      | Chr | Nearest Gene       | Allele | Bilateral neovascular AMD | Unilateral neovascular AMD | P-value OR (95% CI) | P-value* OR (95% CI)* |
|----------|-----|--------------------|--------|--------------------------|----------------------------|---------------------|-----------------------|
| rs7589251 | 2   |                    | G T    | 52 133 136 0.37 58 321 423 0.27 | 6.22 x 10^-4          | 1.56 (1.28–1.91) | 4.16 x 10^-4          |
| rs10208693 | 2   |                    | A G    | 51 138 132 0.37 56 314 433 0.27 | 3.53 x 10^-7          | 1.65 (1.35–2.01) | 4.74 x 10^-7          |
| rs4538253 | 2   |                    | G A    | 58 139 124 0.40 69 344 390 0.30 | 9.49 x 10^-4          | 1.54 (1.26–1.88) | 9.55 x 10^-4          |
| rs7603311 | 2   | STON1-GTF2A11/LHCRG/FSHR | T C    | 48 137 134 0.37 54 311 438 0.26 | 9.14 x 10^-7          | 1.63 (1.33–2.00) | 1.35 x 10^-4          |
| rs4482537 | 2   |                    | C T    | 52 142 121 0.39 60 326 411 0.28 | 3.78 x 10^-7          | 1.65 (1.34–2.02) | 1.36 x 10^-7          |
| rs6545074 | 2   |                    | G T    | 50 142 129 0.38 54 326 420 0.27 | 8.23 x 10^-7          | 1.63 (1.32–2.00) | 2.54 x 10^-7          |
| rs7037739 | 2   |                    | G A    | 48 141 132 0.37 48 325 430 0.26 | 4.73 x 10^-7          | 1.65 (1.34–2.02) | 2.14 x 10^-7          |
| rs900429  | 3   | PLXNA1             | C T    | 8 98 214 0.18 45 329 428 0.26 | 2.96 x 10^-3          | 0.61 (0.48–0.73) | 7.18 x 10^-4          |
| rs1925616 | 10  | CTNNA3             | G A    | 24 129 168 0.28 25 263 515 0.19 | 2.77 x 10^-5          | 1.57 (1.25–1.97) | 8.79 x 10^-4          |
| rs10997482 | 10  |                    | A G    | 24 129 168 0.28 25 263 515 0.19 | 2.77 x 10^-5          | 1.57 (1.25–1.97) | 8.79 x 10^-4          |
| rs2284665 | 10  | ARMS2/HTRA1        | G T    | 43 99 179 0.29 147 359 296 0.41 | 1.39 x 10^-7          | 0.59 (0.48–0.71) | 3.08 x 10^-4          |
| rs8002574 | 13  | LHFP               | T C    | 2 33 285 0.06 12 177 614 0.13 | 2.86 x 10^-4          | 0.43 (0.29–0.63) | 3.84 x 10^-4          |
| rs9525873 | 13  | FLJ38725           | T C    | 49 172 100 0.42 97 327 378 0.32 | 1.77 x 10^-5          | 1.51 (1.25–1.97) | 7.13 x 10^-4          |
| rs8592666 | 13  |                    | T C    | 74 168 79 0.49 135 362 305 0.39 | 2.07 x 10^-5          | 1.49 (1.23–1.80) | 3.86 x 10^-4          |

Table 2. Results of discovery study for the 14 SNPs that showed P-value < 10^-5, comparing bilateral neovascular age-related macular degeneration cases with unilateral cases. SNP, single nucleotide polymorphism; Chr, chromosome; MAF, minor allele frequency; OR, odds ratio; CI, confidence interval.

*Adjusted for age and sex.

Results of the genome-wide association analysis are shown in Fig. 1. Although no chromosomal loci showed genome-wide significance, fourteen SNPs showed P-value < 1.0 x 10^-5; 7 SNPs in STON1-GTF2A11/LHCRG/FSHR region, 1 SNP in PLXNA1, 2 SNPs in CTNNA3, 1 SNP in ARMS2/HTRA1, 1 SNP in LHFP, and 2 SNPs near FLJ38725 (Table 2). No SNPs in CFH showed significant association with bilaterality of neovascular AMD (P > 0.05). Supplementary Table 1 shows the association between the AMD bilaterality and the analyzed 440 SNPs within 10 genes for which associations with AMD were verified in Asian individuals. Although rs11963725 of C2/CFB (P = 0.0124), rs1054060 of C3 (P = 0.0454), rs17130296 of CETP (P = 0.00250), rs4714699 of VEGFA (P = 0.0368), rs6822976 of CFH (P = 0.00256), and rs12638651 of ADAMTS9 (P = 0.00742) showed nominally significant associations, these associations should be interpreted as negative results after permutation tests.
Table 3. Results of two replication studies and meta-analysis for the six loci associated in discovery study, comparing bilateral neovascular age-related macular degeneration cases with unilateral those. SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval. *Adjusted for age and sex.

Table 4. Results of the association study for three loci about the occurrence of AMD, comparing all neovascular age-related macular degeneration cases with Japanese general population cohort. SNP, single nucleotide polymorphism; AF, allele frequency; MAF, minor allele frequency; OR, odds ratio; CI, confidence interval, *Chi-trend.

Discussion

In this current study, we performed GWAS on bilateralty of neovascular AMD for the first time and identified the association of new genetic loci \textit{STONI-GTF2A1L/LHCGR/FSHR} with neovascular AMD bilateralty. ARMS2/HTRA1 could be also associated with bilateralty of neovascular AMD and the association of LHFP should be further investigated.

Regional association plot (Supplementary Figure 1) for the studied SNPs within \textit{STONI-GTF2A1L/LHCGR/FSHR} region did not elucidate which of these genes was correspond to the bilateralty of AMD. \textit{STONI} encodes stonin 1. Although stonin 2 controls synaptic transmission, the function of stonin 1 has not been elucidated\(^9\). \textit{GTF2A1L} is expressed mainly in testis to form a counterpart of general transcription factor IIA.
subunit. In drosophila, transcription factor IIA regulates development of photoreceptor cells. Polymorphisms in STON1-GTF2A1L might promote bilateral development of AMD through their effects on photoreceptor cells synaptic transmission.

LHCGR encodes luteinizing hormone/choriogonadotropin receptor, a receptor for luteinizing hormone (LH) and human chorionic gonadotropin (hCG), and FSHR encodes follicle-stimulating hormone receptor, a receptor for follicle-stimulating hormone (FSH). Both LH and FSH are released from the pituitary gland and stimulate estrogen secretion. Estrogen is associated with inhibition of AMD development and a previous study reported that polymorphisms of estrogen receptor gene were associated with AMD. Aging decreases estrogen production, leading to increased LH/FSH secretion in the elderly via feedback mechanism.

Age-related decrease in estrogen production and increase in LH/FSH secretion are associated, partly, with increased risk of atherosclerosis and heart diseases by affecting lipoprotein/cholesterol metabolism. Considering that several genes in lipoprotein/cholesterol metabolism are associated with AMD development, LHCGR/FSHR would affect the bilaterality of neovascular AMD in part by altering lipoprotein/cholesterol metabolism. Another possibility is that LHCGR/FSHR polymorphism would have localized effect, thereby affecting neovascular AMD bilaterality. Müller cells and retinal pigmented epithelial cells produce hCG and cone photoreceptor cells express its receptor LHCGR. Further study on the roles of LHCGR of photoreceptor cells and the roles of LHCGR/FSHR-induced lipoprotein/cholesterol metabolism alteration would lead to prevention of neovascular AMD development in the fellow eye.

STON1-GTF2A1L/LHCGR/FSHR region also includes long intergenic noncoding RNA (lincRNA, RP11-460M2.1). LincRNA can control gene expression and some lincRNAs might be able to control ocular neovascularization. LincRNA RP11-460M2.1 might have some ability to control bilaterality of neovascular AMD.

Although CFH is a major susceptibility gene for AMD. SNPs in CFH did not have any association with the bilaterality of neovascular AMD in our study. Previous studies also showed that CFH was not associated with the bilaterality of neovascular AMD. In contrast, rs4482537 in LHCGR/FSHR locus had significant association with the bilaterality of neovascular AMD, but not with the occurrence of AMD. On the other hand, previous studies and current study support that ARMS2/HTRA1 is associated with both occurrence and bilaterality of AMD. AMD-associated genes can be classified into three types, genes associated with both AMD occurrence and bilaterality, genes associated with only AMD occurrence, and genes associated with only AMD bilaterality. The second eye involvement in AMD might be regulated by a unique mechanism in addition to the factors associated with the first eye involvement.

LHFP is a HMGIC fusion partner gene in lipoma, one of the most common mesenchymal tumors. LHFP is also associated with mesenchymal differentiation in gliosarcoma. Recent studies suggest that epithelial–mesenchymal transition (EMT) has important roles in the development of AMD. LHFP might affect the bilaterality of neovascular AMD by affecting the EMT process. LHFP is also associated with Alzheimer’s disease that shares common clinical and pathological features with AMD; both Alzheimer’s disease and AMD are preceded by accumulation of amyloid beta. LHFP might affect accumulation of amyloid beta and trigger the second eye involvement of AMD.

Although anti-VEGF treatment has improved treatment outcome of exudative AMD, the SEVEN-UP study reported that 51% of the patients in their study suffered from bilateral neovascular AMD during 7 years of follow-up. The second eye involvement in patients with late AMD is a matter of concern because patients with bilateral late AMD are more prone to visual handicaps than patients with unilateral late AMD. The SEVEN-UP study also suggested that reduced frequency of treatments contributed to the decline of visual acuity. Increasing the frequency of injection might maintain visual acuity for an extended period. Therefore intensive treatment should be initiated in patients with unilateral neovascular AMD who are most likely to develop bilateral neovascular AMD in the future, to maintain good visual acuity. Prediction of the second eye involvement in neovascular AMD would be beneficial for patients with unilateral neovascular AMD. Elucidation of the mechanism to control the second eye involvement might lead to prevention of the second eye involvement.

The limitations of this study are its retrospective nature and relatively small sample size. Studies involving large size might successfully replicate the association of LHFP, and prospective study would further confirm the association of STON1-GTF2A1L/LHCGR/FSHR and ARMS2/HTRA1 to the bilaterality of AMD. Considering that CATT study could not detect any genetic associations between polymorphisms and the second eye involvement within 2 years of follow-up, studies with longer follow-up period should be performed. The unilateral patients included in the current study were significantly younger than the bilateral patients. The unilateral patients may go on to develop bilateral disease with longer follow up, which would reduce the power of detecting genetic associations. Statistical adjustment for the time of diagnosis with unilateral neovascular AMD would also be helpful. Current study was performed only in Asians including both typical AMD and PCV. The prevalence of PCV is higher in Asian than Caucasian. Although the reported prevalence of bilateral involvement is similar between typical AMD and PCV, the ethnic difference cannot be ignored; 40–50% in Caucasian and 10–20% in Asian. Recent studies also suggested the role of ethnic differences in AMD genetic susceptibility. Further study is needed to evaluate whether STON1-GTF2A1L/LHCGR/FSHR and LHFP are associated with neovascular AMD bilaterality in other races.

In conclusion, our GWAS for neovascular AMD bilaterality identified novel genetic loci STON1-GTF2A1L/LHCGR/FSHR and confirmed the association of ARMS2/HTRA1 with the bilaterality. LHFP might also be associated with AMD bilaterality. Prediction of the second eye involvement would be beneficial in determining the long-term management strategy for the first eye of AMD, and elucidation of the mechanisms for the second eye involvement would lead to prevention of the second eye involvement in AMD.
Methods
All procedures used in this study confirmed to the tenets of the Declaration of Helsinki. The Institutional Review Board and the Ethics Committee of each institution approved the experimental protocols; The Kyoto University Graduate School and Faculty of Medicine Ethics Committee, Fukushima Medical University Ethics Committee, Kobe City Medical Center General Hospital Ethics Committee, Singapore National Eye Center Ethics Committee, the Ad hoc Review Board of the Nagahama Cohort Project, and the Nagahama Municipal Review Board of Personal Information Protection. All the participants were fully informed of the purpose and procedures and a written consent was obtained from each.

Study subjects in GWAS for bilaterality. Japanese patients with neovascular AMD were recruited at the Center for Macular Diseases of Kyoto University Hospital (n = 821) and Fukushima Medical University (n = 333) for the discovery stage, and at Kobe City Medical Center General Hospital (n = 170) for the replication stage. Further, patients with neovascular AMD (n = 112) were recruited at Singapore National Eye Center for the second replication stage. All subjects underwent comprehensive ophthalmologic examinations, including dilated contact lens slit-lamp biomicroscopy, fundus photography, fluorescein and indocyanine green angiography (HRA2, Heidelberg Engineering, Heidelberg, Germany), and optical coherence tomography (Spectralis HRA + OCT, Heidelberg Engineering, Heidelberg, Germany).

Neovascular AMD was defined as the presence of exudative AMD as described in the international classification system for age-related maculopathy. Typical AMD involved classic choroidal neovascularization (CNV), occult CNV, or a combination of both. The diagnosis of polypoidal choroidal vasculopathy (PCV) was based on indocyanine green angiography, which showed a branching vascular network terminated in polypoidal lesions. Diagnosis and grading of AMD were performed in a masked manner by two ophthalmologists independently. In cases of disagreement, the third retinal specialist made the final decision. Bilaterality of neovascular AMD and subject age at final visit was used for analysis.

Genotyping. Genomic DNAs were extracted from peripheral blood leukocytes using QuickGene-610L DNA extraction kit (FUJIFILM Co., Tokyo, Japan). Genotyping was performed using Illumina BeadChip, both OmniExpress and HumanExome, HumanOmn12.5–8, or OmniExpress. The distortion of Hardy-Weinberg equilibrium (HWE) was not considered in this study because all samples comprised AMD cases. Stringent quality control, including minor allele frequency (MAF) ≥ 1% and genotype call rate ≥ 95% (per SNP and per individual), was performed using PLINK ver1.07 (http://pangui.mgh.harvard.edu/~purcell/plink/).

Statistical analysis. Association between genotypic distribution of each SNP and the bilaterality of neovascular AMD was examined using logistic regression analysis by adjusting for age and sex using Software R (R Foundation for Statistical Computing, Vienna, Austria). Inflation of the test statistics was assessed using the genomic-control method. SNPs with P-value < 1.0 × 10⁻⁵ were selected as candidates of replication stages. Among the candidate SNPs, one representative SNP was selected from each locus (r² > 0.35). Quality controls were performed using PLINK (ver.1.07; http://pangui.mgh.harvard.edu/~purcell/plink/). For consistent genotyping data across each platform, we performed genomic imputation on available 1000 Genome Project data from East Asian subjects using MACH software (http://www.sph.umich.edu/csg/abecasis/MACH/tour/imputation.html). After imputation, we again performed quality control including MAF cut-offs (>0.01), HWE (P > 1.0 × 10⁻⁶), genotypic success rate (>95%), individual call rate (>99%), and estimated relatedness (PI-HAT < 0.35). Quality controls were performed using PLINK (ver.1.07; http://pangui.mgh.harvard.edu/~purcell/plink/).

Control cohort for the AMD susceptibility test. A fixed dataset of 3,265 unrelated healthy Japanese subjects from the Nagahama prospective genome cohort for the Comprehensive Human Bioscience (The Nagahama Study) was used as a control group in the AMD susceptibility test. In detail, a total of 3,712 individuals from the Nagahama Study were genotyped using HumanHap610K Quad Arrays, HumanOmn12.5 M Arrays, and/or HumanExome Arrays (Illumina Inc., CA, USA). To ensure high-quality genotype data, a series of quality control filters were applied to the data from each platform before imputation, including MAF cut-offs (>0.01), HWE (P > 1.0 × 10⁻⁶), genotypic success rate (>95%), individual call rate (>99%), and estimated relatedness (PI-HAT < 0.35). Quality controls were performed using PLINK (ver.1.07; http://pangui.mgh.harvard.edu/~purcell/plink/). For consistent genotyping data across each platform, we performed genomic imputation on available 1000 Genome Project data from East Asian subjects using MACH software (http://www.sph.umich.edu/csg/abecasis/MACH/tour/imputation.html). After imputation, we again performed quality control including MAF cut-offs (>0.01), HWE (P > 1.0 × 10⁻⁷), genotypic success rate (>90%), individual call rate (>90%), and imputation quality (R² > 0.3).

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Author Contributions
K.K.-K., K.Y., F.M. and N.Y. have designed the study. K.K.-K., K.Y., M.Y., M.M., G.C.-C.-M., Q.F., J.Y.-K., M.S., M.S.-K., M.O., Y.A.-K., I.N., H.H.N., N.G., A.O., H.T., S.O., A.T., Y.K., and T.S. acquired the data. K.K.-K., K.Y., M.Y., M.M., G.C.-C.-M., Q.F., J.Y.-K., F.M., C.-C.K., C.-Y.C., T.-Y.W. and N.Y. analyzed and interpreted data. F.M., C.-C.K., C.-Y.C., T.-Y.W. and N.Y. supervised the study. K.K.-K., K.Y., M.Y., and M.M. wrote the manuscript. All authors reviewed the manuscript.
Additional Information
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