or no drusen.[4] The differences in the genetic background of these two groups have been considered to be one of the reasons for this phenotypic difference.

Recently, a number of susceptible chromosomal loci for AMD have been identified by genome-wide scan using microsatellite markers.[5-9] Direct examinations of the SNPs in these regions showed that three genes, *CFH*,[10] *LOC387715*,[11,12] and *HTRA1*,[13] were associated with AMD in Caucasians and individuals in the Hong Kong population. However, our earlier studies focusing on these regions showed that *LOC387715* (rs10490924) and *HTRA1* (rs11200638) but not *CFH* were associated with the wet-type AMD in the Japanese.[14,15] Furthermore, rs10490924 and rs11200638, which are located on chromosome 10q26, have been shown to be strongly associated with the dry-type,[16] wet-type, [13,16] and the PCV-type of AMD.[17] It is still unclear how these polymorphisms can be strongly associated with the different types of AMD. Further functional studies of both genes at the molecular level are required to decide whether *LOC387715* and/or *HTRA1* are involved in the pathogenesis of the different types of AMD.

With the exception of *CFH*, *LOC387715,* and *HTRA1*, various loci were detected by the GWAS studies.[5-9] These differences may be due to the heterogeneity of the sample population which included both the dry-type and the wet-type AMD patients. To

investigate the involvement of various genetic factors in AMD in more detail, a GWAS was performed using Affymetrix Human Mapping 500K Arrays to genotype over 500,568 tag-SNPs on each selected Japanese patient with only the advanced wet-type AMD.

Methods

Participants

One hundred Japanese patients (average age 74.56 ± 0.88 years) at the advanced stage of AMD (classified as 5b by Seddon et al.[18]) accompanied with a CNV, and two control groups (n = 100 each, average ages 72.01 ± 0.86 years and 69.56 ± 1.23 years) were recruited through the National Hospital Organization and Juntendo University (Table 1). All patients were diagnosed by ophthalmoscopic and fluorescein angiographic findings. In controls, no signs of early AMD, such as soft drusen or irregular pigmentations of the retinal pigment epithelium in the macular area, were observed ophthalmoscopically. An informed consent was obtained from all participants to participate in this study, and the procedures used conformed to the tenets of the Declaration of Helsinki.

Genotyping

DNA was extracted from blood samples using the Magtration System 8Lx (Precision

System Science Co., Ltd., Tokyo, Japan), and 50 ng/μl samples were evaluated by gel electrophoresis. Genechip genotyping was preformed on the Affymetrix GeneChip® Human Mapping 500K Array Set (Affymetrix Japan, Tokyo, Japan) according to the manufacturer's instructions. SNP calling was carried out with the GeneChip Genotyping Analysis software, which uses the Dynamic Model (DM) algorithm, a model-based on a genotype-calling (GC) algorithm. This algorithm generates a QQC call rate for each array. Final GCs were obtained using the Bayesian Robust Linear Model with the Mahalanobis distance classifier (BRLMM) algorithm, implemented in the BRLMM Analysis Tool (BAT) available at the Affymetrix web site.

Statistical analyses

Statistical analyses were carried out with the R version of 2.6.2 (R Development Core Team (2008). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0). The deviation of the genotype distributions from Hardy-Weinberg equilibrium (HWE) was determined with Pearson's chi square test for the case and control samples. All genotyped data sets were filtered individually and then merged. SNPs with >10% missing data, HWE *P* values < 0.0001, and minor allelic frequency < 5% were excluded. For each case-control study,

Fisher's exact test was used to compare the allelic or genotypic frequencies in three different models of each case group with controls. In the allelic model, the allelic frequencies were compared between cases and controls using a 2 x 2 contingency table. In the first genotypic model, the frequencies of the three genotypes were compared using a 2 x 3 contingency table, in the second model, the frequencies of the homozygote for the minor allele were compared using a 2 x 2 contingency table, and in the third model, the frequencies of the homozygote for the major allele were compared using a 2 x 2 contingency table. The Cochran-Armitage trend test was used in the first genotypic model. The odds ratio (OR) and 95% confidence interval (CI) were calculated by Woolf's method. Haplotype blocks were determined using Haploview version 4.0 with all parameters set at the default values. The Benjamini-Hochberg method was used to identify SNPs significantly associated with AMD.

Results

Genome-wide association analysis (GWAS)

We conducted a GWAS on the DNA obtained from Japanese patients with late stage wet-type AMD. To increase the power of detecting genetic defects, we used only cases of AMD at stage 5b in at least one eye. Although the controls were age-matched (Table 1),

the gender of the population was biased because of the higher incidence of men which is known to be a characteristic of the Japanese population.[4]

We genotyped 500,568 tag-SNPs using the Affymetrix Human Mapping 500K Array Set in the 100 AMD cases and 200 controls. For statistical analysis, we selected 329,556 tag-SNPs which passed all of the quality control filters (HWE $P < 0.0001$, minor allele frequency < 5%, call rate < 90%) to reduce the number of false-positive signals. The 4,795 SNPs with genotypic P values < 0.01 are plotted in Figure 1. Four hundred and fifty-five SNPs associated with AMD with genotypic P values < 0.001 are shown in the Supplementary Material, Table S1.

GWAS identified several genomic locations as being potentially associated with AMD risk. Ten SNPs, rs10490924, rs3750848, rs2672587, rs2874794, rs12462443, rs2714212, rs9599819, rs3763022, rs12595534, and rs10510110, had a minimum genotypic P values of $<10^{-6}$ (1.19×10^{-13}, 1.38×10^{-13}, 8.02×10^{-9}, 3.05×10^{-6}, 3.70×10^{-6}, 4.04×10^{-6}, 5.08×10^{-6}, 5.59×10^{-6}, 5.98×10^{-6} and 9.50×10^{-6}, respectively, Table 2). The maximum ORs of the ten SNPs were 8.29, 8.24, 4.63, 3.93, 3.94, 2.88, 1.73, 6.62, 8.91, and 3.09 respectively (Ttable 2). Among the ten SNPs, rs10490924, rs3750848, and rs2672587 had a with false discovery rate of < 0.05 (Benjamini-Hochberg method), and were determined to be significantly associated with the advanced wet-type AMD in Japanese

patients (fig. 2).

Haplotype block analyses

The leading two SNPs, rs10490924 and rs3750848 which have been reported to be associated with AMD,[12] map to a small haplotype block on chromosome 10q26 (Fdig. 3). rs10490924 and rs3750848 are located within exon 1 and intron 1 onf the *LOC387715* gene in this region. Another SNP, rs2672587, is located in the intronic region of the *HTRA1* gene between blocks two and three, and is located downstream of the leading two SNPs (Ffig. 3).

Discussion

We have performed a GWAS on Japanese patients with advanced wet-type AMD using Affymetrix GeneChip® Human Mapping 500K Array. Surprisingly, only three tag-SNPs, located oin the the 10q26 (*LOC387715/HTRA1)* locus, were significantly associated with advanced wet-type AMD, while several candidate loci (6q14, 19q13, 2q22, 13q21, 5q33 and 15q13) appeared for AMD susceptibility. Logically, further data accumulation is neededdemanded, and the genetic homogeneity of the Japanese population [19] is an advantage of this study. Some of the candidate loci may be specific to the Japanese

population. Previous studies using microsatellite markers [5,7,8,9] or Affymetrix 100K array [20] have shown that 10q26 and 1q32 are the two most significantly associated loci for AMD. Although our results on 10q26 are in good agreement with these earlier observations, no association was detected for 1q32. This is consistent with our earlier study and others which showed that the *CFH* gene located on 1q32, one of the major risk factor of AMD,[10] is not responsible for advanced wet-type AMD in the Japanese.[14] Taking into consideration that these same characteristics above wereas also reported forby a Chinese population,[21] our GWAS results may have revealed some of the common features common to the east Aasian. Furthermore, the results from Zhang et al pointed out that the *LOC387715/HTRA1* region is more strongly associated with the wet-type AMD than *CFH* region.[20] TAlthough both regions are responsible for the wet-type and dry-type AMD, a lack of *CFH* risk may be one of the reason for which explains the low appearance of the dry type AMD in the Japanese.

The haplotype block which contain two of the associated SNPs, rs10490924 and rs3750848 at the 10q26 locus, include the *LOC387715* gene (Ttable 2 and Ffig. 3). *LOC387715* has been reported to be one of the most potent genetic risk factors for both the wet- and dry-type AMD.[11,12,17,22] This gene appears to have been recently evolved in the primate lineage.[11] Although the function of this gene is not known, Kanda

et al have shown that the *LOC387715* gene product is located in the mitochondria of COS-1 transfected cells.[12] They also investigated the effect of amino acid substitution caused by rs10490924 (A69S), but no difference was found between the COS-1 cells transfected with the wild type or the mutant type *LOC387715*. More recently, Fritsche et al detected the LOC387715 protein in human placenta and retina by immunohistochemistry.[22] Immunohistochemical analyses showed that the expression of LOC387715 was in the mitochondria-enriched inner segments of the retina, thus indicating the possibility of LOC387715 contributing to mitochondrial function. Further studies are needed to determine the exact role played by LOC387715 in the onset of AMD.

HTRA1, another candidate gene, was located approximately 6 Kbp downstream of *LOC387715*. *HTRA1* encodes a serine protease which belongs to the high temperature requirement factor A family and is conserved widely from prokaryotes to humans.[23] Prokaryotic HTRAs are involved in protein quality control, e.g., repair and degradation of misfolded proteins,[23] and human HTRAs are believed to be involved in arthritis, apoptosis, neuromuscular disorders, and cancer although the underlying biological mechanisms are not well understood.[24] rs2672587is aAn intronic SNP rs2672587

within this region was significantly associated with AMD in this study (table 2).

rs11200638, a SNP located upstream of rs2672587, has been reported to be strongly associated with AMD.[13,15,17] rs11200638 is located 512 bp upstream of the transcription initiation point within the putative AP2 binding sites. *In vitro* and *in vivo* data from two laboratories have reported that this polymorphism leads to a transcriptional up-regulation of *HTRA1* and sequentially triggers the onset of AMD.[13] Chan et al detected an up-regulation of *HTRA1* in the macular lesions of AMD using immunohistochemical analysis.[25] Grau et al demonstrated that the amyloid precursor protein (APP), a precursor of amyloid beta (Aß) which is a major hallmark of Alzheimer's disease (AD), is one of the native substrates of HTRA1.[26] Aß has been shown to exist in substructures of drusen in the retinas of AMD patients but not in the drusen from controls.[27] Several studies have shown that localized chronic inflammation triggered by complement activation in AD and AMD can be attributed to Aß.[1] It has also been suggested that the accumulation Aß affects the balance of VEGF and pigment epithelium-derived factor (PEDF) in the RPE, which may lead to the CNV in eyes with AMD. [28] Furthermore, anti-Aß antibody has been proven to be effective asfor immunotherapies of AMD.[29] Although a rs11200638-dependent up-regulation of

HTRA1 has been negated by others,[12] it is still unclear whether *LOC387715* or/and *HTRA1* is a genetic risk(s) for AMD on 10q26.

Unfortunately, rs11200638 was absent from the Affymetrix Human Mapping 500K Array Set. Thus, we have genotyped rs11200638 separately by sequencing analysis (data not shown). rs11200638 was included in block two together with rs10490924, and separately from rs2672587 (data not shown), i.e., the most strongly associating haplotype block includes gene regions for both *LOC387715* and *HTRA1*.

The remaining seven SNPs in Table 2 appear to be non-significant (fFig. 2), although additional case-control analysis may be neccessary. Amongst them, rs2714212 is located in intron 3 of the low density lipoprotein-related protein 1B (*LRP1B*) gene on chromosome 2 (data not shown). LRP1B is a member of the low-density lipoprotein receptor family.[30] Together with its closely related receptor LRP1, LRP1B has been suggested to be involved in Aß production in AD.[30] rs3763022 is located in the 3'-UTR region of *SH3TC2* gene on chromosome 5 (data not shown). A mutation in the *SH3TC2* gene mutation ishas been known tothat lead to inherited motor and sensory neuropathies (HMSN), also called Charcot-Marie-Tooth disease (CMT). rs10510110 is located together with *PLEKHA1*, a gene existing approximately 20 Kbp upstreams of *LOC387715*,[11] on block 1 of 10q26 locus (Ffig. 3). No gene or RNA have been registered on databases

within the corresponding haplotype blocks on 6q14, 19q13, 13q21, and 15q12 in which rs2874794, rs12462443, rs9599819 and rs1295534 are located (data not shown). However, there is a possibility of the existence of unknown mechanisms related to these regions. These loci may be critical for the development of AMD in combination with *LOC387715* (rs10490924) and/or *HTRA1* (rs11200638), and other behavioral, nutritional, and environmental factors. Further investigations are required of the individual regions to determine the molecular mechanisms related to the pathogenesis of AMD.

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Supplemental material

Supplemental Table 1

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Figure legends

Figure 1. Genome-wide association study for wet -type AMD susceptibility loci.

The analyseis compared 100 stage 5b AMD cases with 200 population-based controls (stage three analysis). The x-axis represents genomic locations, and the y-axis shows $-\log_{10}$ (genotypic *P*-value). All of the SNPs on autosomal chromosomes with genotypic *P* values < 0.01 are plotted.

Figure 2. Determination of statistical significant SNPs.

The Benjamini-Hochberg method to correct for multiple testing was used to identify SNPs significantly associated with AMD. A false discovery rate of 0.05 was used to determine statistical significance.

Figure 3. Haplotype block structure of the AMD-associated regions.

Squares shaded pink or red indicate significant linkage disequilibrium between SNP pairs (bright red indicates pairwise $D' = 1$), white squares indicate no evidence of significant linkage disequilibrium, and blue squares indicate pairwise $D' = 1$ without statistical significance. Locations of the regions on each chromosome are shown in scales above.

Genes within the views are shown by arrows. SNPs with a genotypic *P*- value < 10^{-6} in stage three analysis are indicated by open arrowheads. Haplotype block including these SNPs are surrounded with bold lines.

(A.) Haplotype block patterns on chromosome 10. rs10490924 and rs3750848 waere included in block two within the *LOC387715* gene region. rs2672587 is located between block two and three. rs10510110 is located on block one together with the *PLEKHA1* gene.