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https://doi.org/10.15017/21724

出版情報：九州大学, 2011, 博士（医学）, 課程博士
バージョン：
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Genome-wide association study identifies two susceptibility loci for exudative age-related macular degeneration in the Japanese population

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Introductory paragraph

Age-related macular degeneration (AMD), the leading cause of irreversible blindness in the world, is a complex disease caused by multiple environmental and genetic risk factors. To identify genetic factors that modify the risk of exudative AMD in Japanese, we conducted a GWAS and a replication study using a total of 1,536 cases with exudative AMD and 18,894 controls. In addition to *CFH* (rs800292, $P = 4.23 \times 10^{-15}$) and *ARMS2* (rs3750847, $P = 8.67 \times 10^{-29}$) loci, we identified two new susceptibility loci for exudative AMD: *TNFRSF10A-LOC389641* on chromosome 8p21 (rs13278062, combined $P = 1.03 \times 10^{-12}$, OR = 0.73) and *REST-C4orf14-POLR2B-IGFBP7* on chromosome 4q12 (rs1713985, combined $P = 2.34 \times 10^{-8}$, OR = 1.30). Fine mapping revealed that rs13278062, which is known to alter *TNFRSF10A* transcriptional activity, showed the most significant association in 8p21 region. Our results provide new insights into the pathophysiology of exudative AMD.

(147 Words)

Main text

Age-related macular degeneration (AMD) is a major cause of severe visual impairment for elderly population in developed countries[1,2]. Late AMD is divided into exudative AMD and geographic atrophy (GA), and the prevalence of these types are different between European and Asian population[3,4]. Exudative AMD is a major type of late AMD in Asian population and is characterized by abnormal vasculopathies arising from the choroidal vasculature which may lead to recurrent serous exudation and hemorrhages[3]. In contrast, GA is a common type of late AMD in European population and is characterized by retinal pigment epithelium (RPE) atrophy and thinning of the retina without exudative or hemorrhagic changes. Although the inflammation of RPE-choroid interface and the apoptosis of photoreceptor and RPE cells have crucial role for the development of AMD, the precise pathogenesis of AMD is not fully elucidated[5,6].

Previous genome-wide association studies (GWAS) have identified many common variants on AMD risk[7-12]. Landmark GWAS identified complement factor H (*CFH*) and age-related maculopathy susceptibility 2 (*ARMS2*) as the susceptibility genes for AMD[7,8]. Recent advances in genetic research have clarified that the variants of several complement pathway-associated genes have important roles in the pathogenesis of AMD[13-17]. Although GWAS have identified 8 susceptibility loci for AMD, most of these findings were come from the results of European population[7-11]. Regardless of the differences in the prevalence of AMD type between European and Asian population, there is scarce information for the susceptibility genes of AMD in Asian population.

To investigate the genetic background of exudative AMD in the Japanese population, we conducted a GWAS to identify genes related to exudative AMD

susceptibility using 827 cases and 3,323 controls. These samples were genotyped using the Illumina Human610-Quad BeadChip in cases and the Illumina HumanHap550v3 BeadChip in controls. Genotype concordance between these two BeadChips was 99.99% among 182 duplicate samples, indicating a low possibility of genotype error. After we applied stringent quality control criteria, we carried out association analysis in 457,489 autosomal SNPs that were available on both BeadChips. Principal component analysis (PCA) showed no population structure and quantile-quantile plot showed that the inflation factor was 1.057 (**Supplementary Fig. 1a and 1b**). To further examine the possibility of population substructure and its influence on our GWAS results, we performed PCA again only using HapMap JPT and CHB as the references. Almost all subjects fell into the known two main clusters of the Japanese population (**Supplementary Fig. 1c**). When we evaluated the quantile-quantile plot only using the samples in the main (Hondo) cluster, the inflation factor was 1.076 (**Supplementary Fig. 1d**). Therefore, we considered that genotype misclassification or population substructure might not be the cause of the difference in the inflation factor.

In our GWAS, two loci reached a genome-wide significance level of association ($P < 5 \times 10^{-8}$, **Supplementary Fig. 1g**). These loci were already reported in previous GWAS[7-12]; *ARMS2* (rs3750847, $P = 8.67 \times 10^{-29}$) and *CFH* (rs800292, $P = 4.23 \times 10^{-15}$). Results of these loci were shown in **Supplementary Fig. 2**. We also checked the association of reported susceptibility loci for AMD (**Supplementary Table 1**). We found significant association in three loci (*CFI*, *C2*, and *CFB*), whereas susceptibility loci identified in recent GWAS of European population (*TIMP3* and *LIPC*) were not replicated probably due to the lower statistical power. Interestingly, rs2230199, a marker SNP at *C3* locus, was not polymorphic in Japanese. Although exudative AMD

is a major type in Japanese compared to European, these results indicate that the underlying disease mechanisms of AMD are largely similar for both populations, and the differences in genetic modifiers or environmental factors may represent the differences in the prevalence of a specific late stage AMD type.

To identify additional susceptibility loci, we conducted a replication study using an independent set of 709 Japanese exudative AMD cases and 15,571 controls. Among 146 SNPs that showed P value of $< 1.0 \times 10^{-4}$ in GWAS, we selected 77 SNPs for the replication study after excluding 47 SNPs within the same locus ($r^2 > 0.8$) and 22 SNPs located at previously reported loci. We successfully genotyped all 77 SNPs using the multiplex PCR-based Invader assay and found significant association in two SNPs after Bonferroni correction (corrected $P < 6.49 \times 10^{-4}$; **Supplementary Table 2**). When we combined the results of GWAS and a replication study by the inverse variance method, these two SNPs reached a genome-wide significance level of association: rs13278062 on chromosome 8p21 (combined $P = 1.03 \times 10^{-12}$, OR = 0.73, 95% CI 0.67 - 0.80) and rs1713985 on chromosome 4q12 (combined $P = 2.34 \times 10^{-8}$, OR = 1.30, 95% CI 1.19 - 1.42, **Table 1**). Odds ratios were quite similar and we did not observe any heterogeneity across the studies. Previous linkage study has also indicated the association on chromosome 8p21[18].

Since exudative AMD is classified into typical AMD (t-AMD) and polypoidal choroidal vasculopathy (PCV), we also assessed the effect of two SNPs on the susceptibility of AMD subtypes (**Supplementary Table 3**). Both SNPs showed similar effect on either subtypes, but the risk of rs1713985 was relatively high for t-AMD. Although we performed age-adjusted analysis, there is a possibility that aging or cohort effect might distort the findings of our study because controls were younger than cases. However, we did not find significant differences in the minor allele

frequencies (MAF) of two SNPs in every 10-year age groups (**Supplementary Table 4**).

To narrow down the candidate regions and to identify susceptibility genes for exudative AMD, we carried out fine mapping using GWAS case-control samples. We first constructed linkage disequilibrium (LD) block and the - $\log_{10}$ (p-value) plot of chromosome 8p21 using GWAS data. We found that the most associated SNP (rs13278062) represented an LD block that spanned from 23.078 Mb to 23.152 Mb. Then, we selected and genotyped 18 SNPs around this LD block based on the data from the HapMap phase II Japanese population with MAF of ≥ 0.05. Next, we re-sequenced a 51-kb region from 23.094 Mb to 23.145 Mb using 48 individuals with exudative AMD. After excluding repeat sequences, we identified 9 new SNPs in addition to the 88 known SNPs registered at dbSNP database. After excluding 23 SNPs already genotyped, we genotyped 74 SNPs with MAF of ≥ 0.05 and succeeded in 73 SNPs. However, no SNPs showed stronger association than rs13278062 (**Figure 1a and Supplementary Table 5a**). We also performed haplotype analysis using the four highly associated SNPs (rs2235126, rs7820465, rs13278062, and rs13281363), however, no haplotype showed stronger association than the single-marker association of rs13278062 (**Supplementary Fig. 3**).

Rs13278062 was located in *LOC389641* gene and also located at 397 base pairs upstream of the tumor necrosis factor receptor superfamily 10a (*TNFRSF10A*) which encodes one of the TRAIL receptors, TRAILR1. TRAILR1 is broadly expressed including human adult RPE[19] and rod photoreceptors in mice[20], whereas the expression of LOC389641 was low or absent according to Gene Expression Omnibus (GEO) database. Binding of TRAIL to TRAILR1 is known to induce apoptosis via caspase 8 activation[21]. In addition, TRAIL/ TRAILR1 complex has

non-apoptotic pathway that induces the production of inflammatory cytokines and the promotion of inflammation through activation of nuclear factorκB [22,23]. Previous study showed that the activator protein 1 will bind the sequence around rs13278062 and directly regulate TRAILR1 mRNA expression[24]. Moreover, the G allele of rs13278062 is reported to enhance the transcriptional activity of TRAILR1 by 1.2 to 1.5-fold as compared to T allele[25]. Although further functional studies are needed, these results speculate that *TNFRSF10A* will be the susceptibility gene for exudative AMD and rs13278062 may be the candidate of functional significance.

The second most significant association was observed at rs1713985 on chromosome 4q12. Based on the LD block and the - $\log_{10}$ (p-value) plot of chromosome 4q12 obtained by GWAS data, we found that rs1713985 represents an LD block that spanned from 57.421 Mb to 57.611 Mb, which includes four genes, *REST, C4orf14, POLR2B* and *IGFBP7* (**Fig. 1b**). Then, we selected and genotyped 120 SNPs around this LD block based on the data from the HapMap phase II Japanese population with MAF of ≥ 0.05 (**Supplementary Table 5b**). However, this analysis could not narrow down the candidate region because of the long range LD. According to the global expression profiles in GEO database, all four genes were expressed in human adult RPE[19] and rod photoreceptors in mice[20]. *REST* is a transcriptional repressor that may act as a master negative regulator of neurogeneisis. Overexpression of *C4orf14* gene product is reported to induce apoptosis by regulating mitochondrial nitric oxide and calcium[26]. *IGFBP7* gene product, angiomodulin, is reported to bind chemokines and growth factors including vascular endothelial growth factor A, whose expression is high in RPE cells of AMD.

In conclusion, our data showed that *TNFRSF10A-LOC389641* on chromosome 8p21 and *REST-C4orf14-POLR2B-IGFBP7* on chromosome 4q12 are new

susceptibility loci for exudative AMD in the Japanese population. Futher functional studies are neccesary to clarify the mechanisms of these loci on the susceptibility to exudative AMD.

(1,485 Words)

URLs. GEO, http://www.ncbi.nlm.nih.gov/geo/; R statistical environment version 2.7.0, http://www. R-project.org/; PLINK 1.05, http://pngu.mgh.harvard.edu/~purcell/plink/; Genetic Power Calculator, http://pngu.mgh.harvard.edu/~purcell/gpc/.

Accession codes. The expression microarray data on the human adult RPE and rod photoreceptors in mice have been deposited in the GEO database under accession number GSE18811 and GSE22317.

ACKNOWLEDGMENTS

We thank all of the patients who participated in this study. We are grateful to A. Yoshida, K. Kano, S. Kawahara, R. Arita, K. Ishikawa, E. Hasegawa, R. Asato, S. Notomi, T. Asakuma, and A. Kuni of the Kyushu University and K. Horie-Inoue, S. Inoue, and T. Awata of the Saitama Medical University and H. Bessho, N. Kondo, and W. Matsumiya of the Kobe university and M. Inoue of the Yokohama City University Medical Center for collecting samples. We thank the staff of the Laboratory for Genotyping Development, Center for Genomic Medicine, RIKEN, staffs of the BioBank Japan project, and the members of the Rotary Club of Osaka-Midosuji District 2660 Rotary International in Japan. We want to express special thanks to F. Miya for the support of gene expression data. This work was conducted as a part of the BioBank Japan Project and supported by the Ministry of Education, Culture, Sports, Sciences and Technology of the Japanese government.

AUTHOR CONTRIBUTIONS

S.A., T.I., Y.N. and M.K. designed the study; S.A., N.H., K.A., T.A., and M.K. performed genotyping; S.A. and M.K. wrote the manuscript; A.T. performed statistical analysis at the genome-wide phase; Y.N. and M.K. managed DNA samples belong to BioBank Japan; T.I. and Y.N. obtained funding for the study. M.Y., Y.O., S.Y., and H.E. collected GWAS samples; T.T., K.M., S.H., A.N., A.A., and K.K. collected case samples for replication study; Y.K., N.K., Y.N., and M.K. supervised the study.

COMPETING FINANCIAL INTEREST

The authors declare no competing financial interests.

FIGURE LEGEND

Figure 1. Case-control association plots, LD map and genomic structure of the *TNFRSF10A- LOC389641* region in chromosome 8p21 (a) and the *REST- C4orf14-POLR2B-IGFBP7* region in chromosome 4q12 (b). Candidate region is shown between two black dotted lines. Fine mapping was performed in the region from 23.09 to 23.14 Mb in chromosome 8p21 and 57.42 to 57.61 Mb in chromosome 4q12. Blue diamonds represent $-\log_{10} P$ obtained from GWAS and fine mapping. The LD map based on *D'* values was drawn using the genotype data of the cases and controls in GWAS samples. Blue line indicates the position of marker SNP (rs13278062 (a), rs1713985 (b)).

Table 1. Summary of the GWAS and replication study.

SNP	Allele	Minor allele	Chr	Chr Loc	Gene	Study	No. of samples		MAF		Age, and sex-adjusted			P_{het}
							Case	Ctrl	Case	Ctrl	*P* value	OR	95% CI	
rs13278062	T/G	T	8	23,138,916	*TNFRSF10A-LOC389641*	GWAS	827	3,323	0.417	0.343	2.46E-06	0.71	(0.62-0.82)	
						Replication	701	15,565	0.417	0.346	8.19E-08	0.74	(0.66-0.82)	
						Combined					1.03E-12	0.73	(0.67-0.80)	0.68
rs1713985	T/G	G	4	57,481,207	*REST-C4orf14-POLR2B-IGFBP7*	GWAS	827	3,323	0.333	0.286	9.03E-05	1.34	(1.16-1.56)	
						Replication	708	15,569	0.329	0.282	5.71E-05	1.27	(1.13-1.43)	
						Combined					2.34E-08	1.30	(1.19-1.42)	0.56

Age and sex-adjusted *P* value was calculated by logistic regression analysis under additive model. Combined *P* value was calculated by the inverse variance method.
P values of heterogeneities across the population were estimated formally by using Cochran's Q test. Chr; chromosome, Loc; location, MAF; minor allele frequency, OR; odds ratio, CI; confidence interval.

ONLINE METHODS

Samples. Characteristics of each study subjects are shown in **Supplementary Table 6**. For GWAS, 827 individuals with exudative AMD were collected at Kyushu University. The diagnosis of exudative AMD was based on comprehensive ophthalmic examination, including fluorescein angiography (FA) findings and indocyanine green angiography (ICGA) findings and optical coherence tomography (OCT) after pupil dilation. We classified exudative AMD into 4 subtypes under the established criteria[27-30]: typical neovascular AMD (t-AMD), polypoidal choroidal vasculopathy (PCV), retinal angiomatous proliferation (RAP) and unclassified. In 827 GWAS cases, we found 298 cases with t-AMD, 480 with PCV, 14 with RAP and 23 unclassified cases. Among these, we found 12 patients who had different subtypes in each eye. Patients with other macular diseases such as high myopia, angioid streaks, and central serous chorioretinopathy were excluded. For the control subjects, we used genome-wide screening data of BioBank Japan which consists of 2,421 individuals with thirteen diseases and 902 healthy volunteers recruited from Osaka-Midousuji Rotary Club, Osaka, Japan[31].

For the replication study, 709 individuals with exudative AMD were recruited at Saitama Medical University (n = 396), Kobe University (n = 212), and Yokohama City University Medical Center (n = 101) under the same criteria as GWAS cases. The numbers of exudative AMD subtypes were 325 for t-AMD, 358 for PCV, 3 for RAP, and 23 for unclassified subtype. We also used genome-wide screening data of BioBank Japan which consists of 15,571 individuals with ten diseases (colorectal cancer, breast cancer, prostate cancer, lung cancer, stomach cancer, diabetes, arteriosclerosis obliterans, atrial fibrillation, cerebral infarction, and myocardial infarction) as controls.

All control individuals had not had any eye examination, therefore it is unknown what ocular conditions, including AMD, are present. Moreover, controls were significantly younger than cases which indicate that some of the controls will go onto develop AMD as they age, although the incidence of late AMD is low in the Japanese population[32]. Since these limitations will underestimate the impact of SNPs on the development of late AMD, true associations may be stronger than that shown in this study.

All participants provided written informed consent to this study. This study was approved by the ethical committees of Kyushu University, Saitama Medical University, Kobe University, Yokohama City University Medical Center, Institute of Medical Science. University of Tokyo and RIKEN Yokohama Institute.

SNP genotyping. For the GWAS, we genotyped 832 exudative AMD patients using Illumina Human610-Quad BeadChip and 3,323 controls using the Illumina HumanHap550v3 BeadChip. Although call rate was ≥ 0.98 for all cases and controls, five cases were excluded due to pair closely related samples. Among the common SNPs in both BeadChips, 457,489 SNPs in autosomal chromosomes passed the quality control filters (call rate of ≥ 0.99 in both cases and controls and *P* value of Hardy-Weinberg equilibrium test of $\geq 1.0 \times 10^{-6}$ in controls) and were further analyzed. For the replication study, we selected 146 SNPs that showed an age, and sex-adjusted *P* value of $< 1.0 \times 10^{-4}$ in the GWAS. Among them, 22 SNPs were located at previously reported loci[7-12] and excluded from further study. We calculated the LD coefficient (r^2) between the remaining SNPs, and selected the 77 SNPs with the lowest *P* value within each r^2 of ≥ 0.8. In the replication study, we genotyped an additional panel of 709 exudative AMD patients using the multiplex PCR-based

Invader assay[33] (Third Wave Technologies). We regarded genotyping as success when the number of undetermined samples was less than 10 in a 384-well plate.

Fine mapping and re-sequencing. For the fine mapping, we used all case and control samples in the GWAS. Tagging SNPs were selected from MAFs of 5% or higher in the region of interest based on the HapMap phase II JPT population. Re-sequencing of candidate regions was performed in 48 exudative AMD cases by using an ABI3730 Genetic Analyzer.

Statistical analysis. In all stages, associations of each SNP were assessed by age- and sex-adjusted logistic regression analysis under an additive model. Combined analysis of the GWAS and the replication study was conducted using the inverse variance method. Heterogeneities across the population were estimated formally by using Cochran's *Q* test. To characterize the population structure, we computed genome-wide average identity by state and performed PCA using the EIGENSTRAT program. GWAS and replication data were calculated using R statistical environment version 2.7.0 or PLINK 1.05 software[34]. Haploview software was used to analyze LD values[35].

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