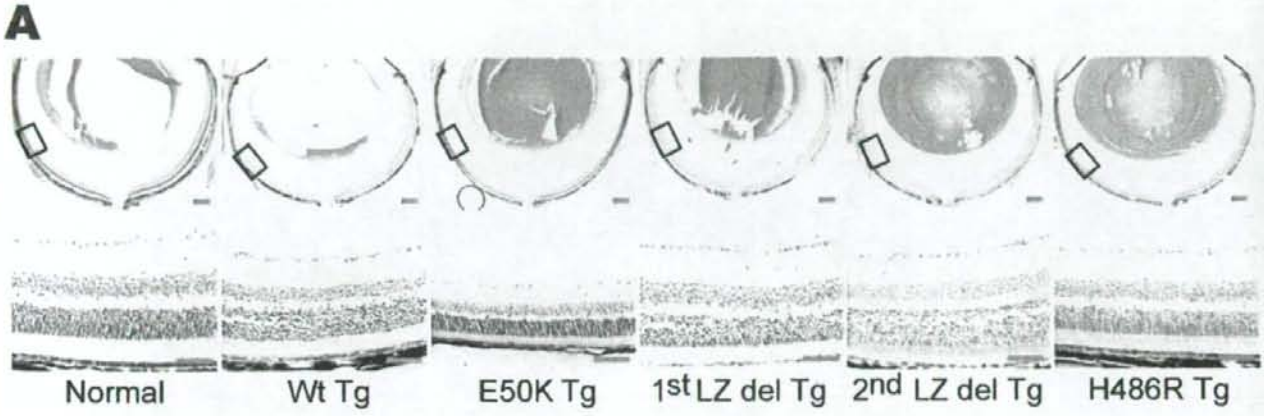
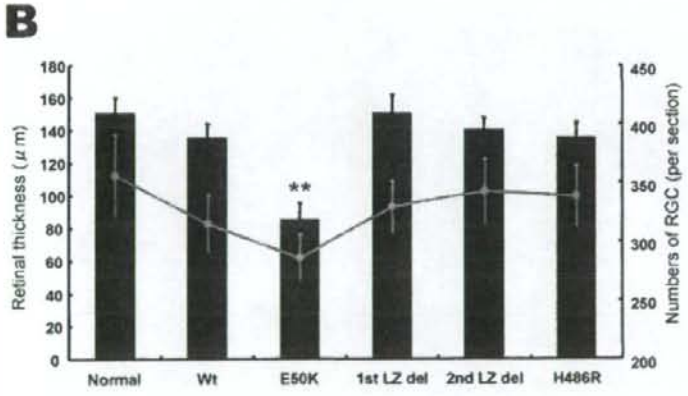
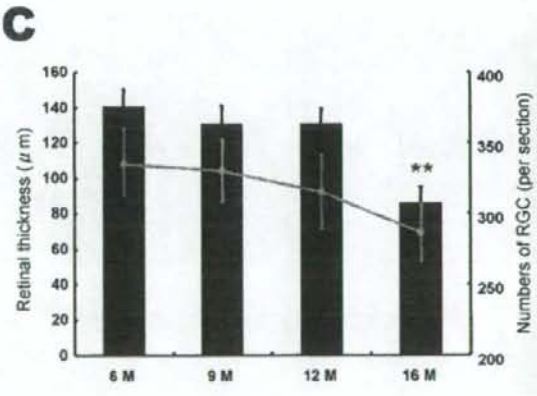
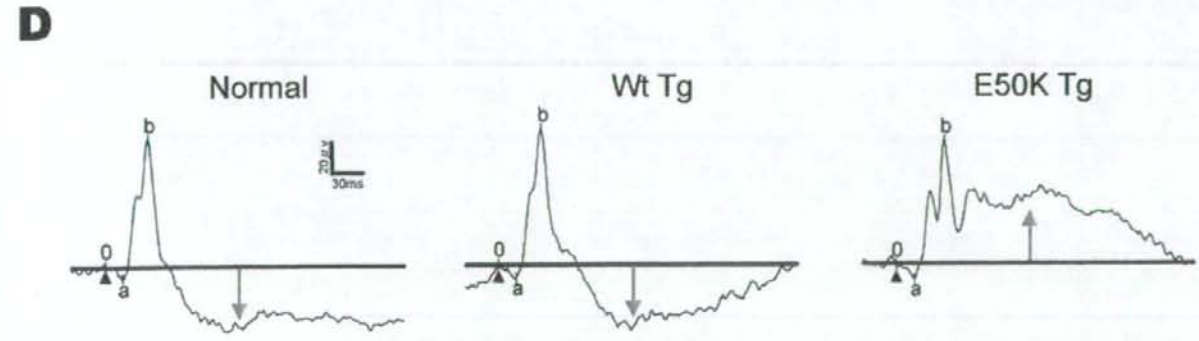
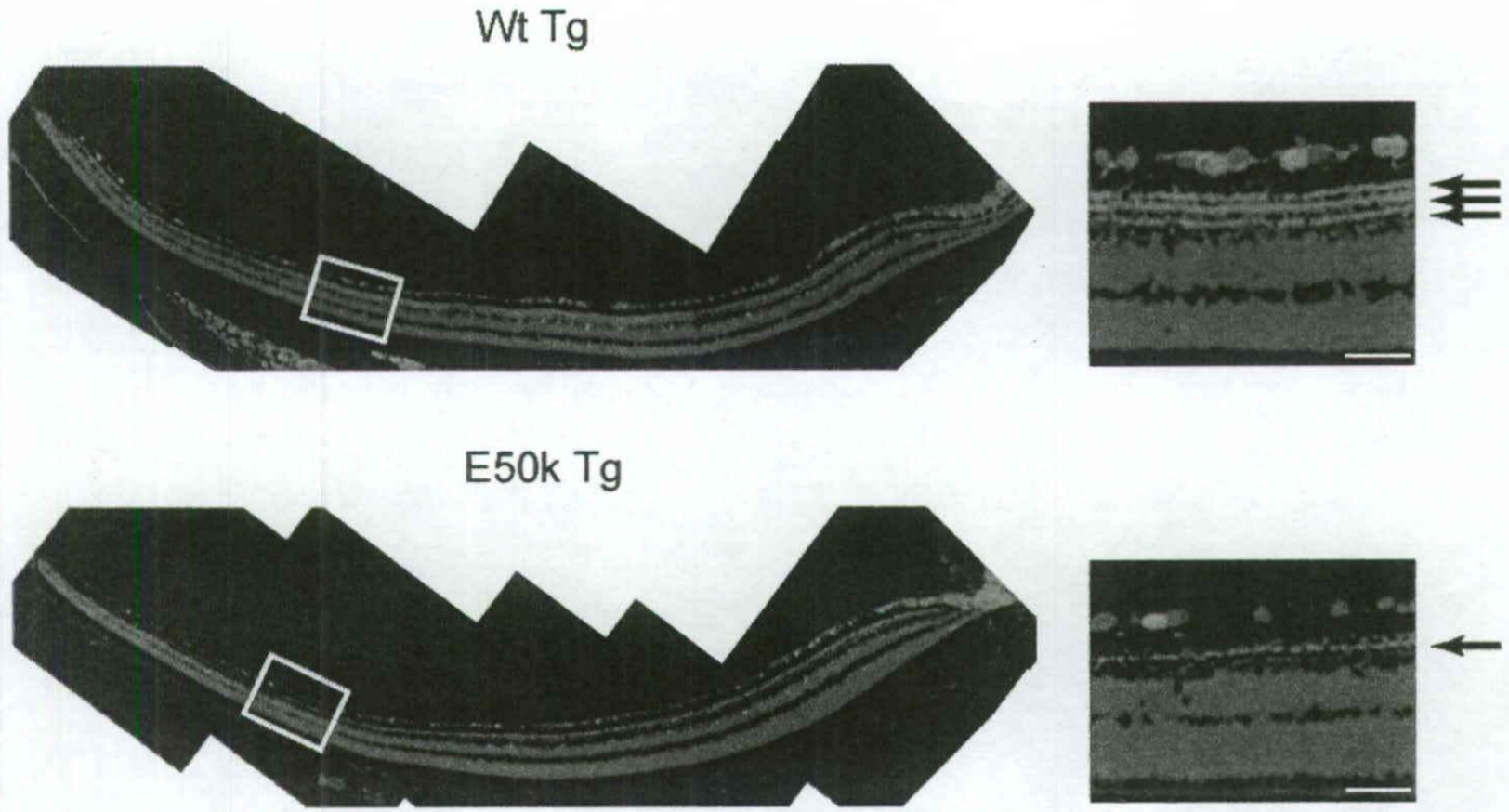

A
Normal
Wt Tg
E50K Tg
1st LZ del Tg
2nd LZ del Tg
H486R Tg


B
Retinal thickness (μm)
Numbers of RGC (per section)
**
Normal
Wt
E50K
1st LZ del
2nd LZ del
H486R

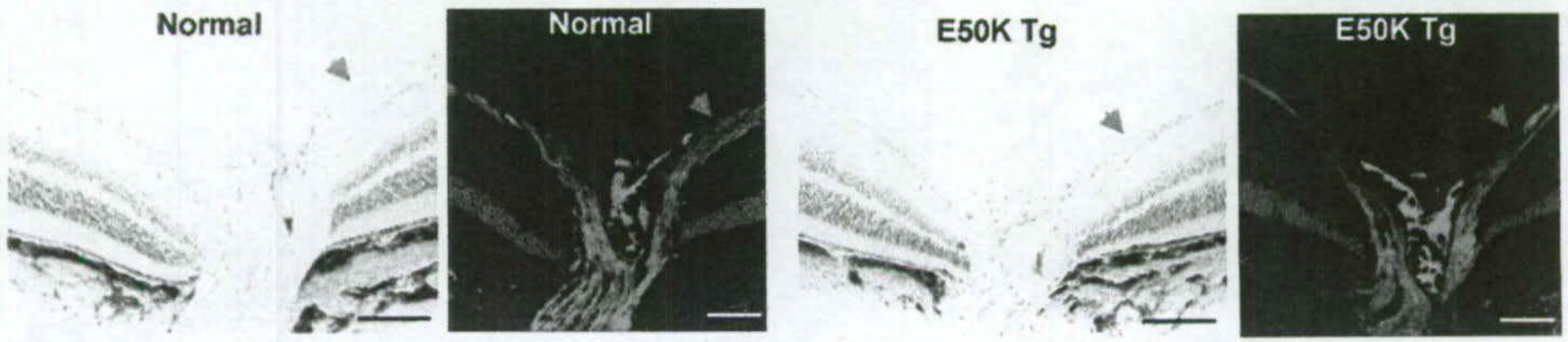

C
Retinal thickness (μm)
Numbers of RGC (per section)
**
6 M
9 M
12 M
16 M

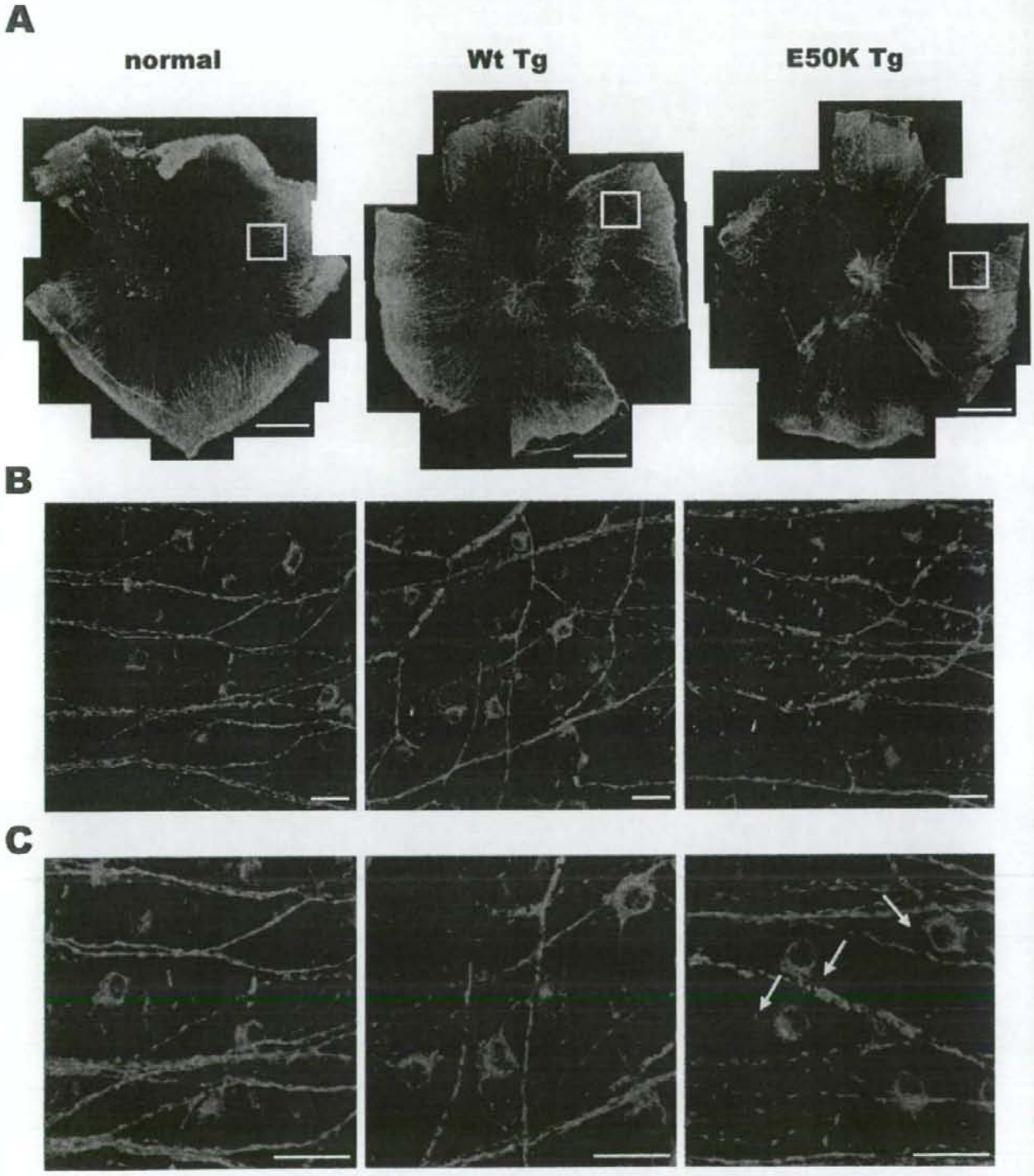

D
Normal
Wt Tg
E50K Tg
20μV
30ms
0
a
b

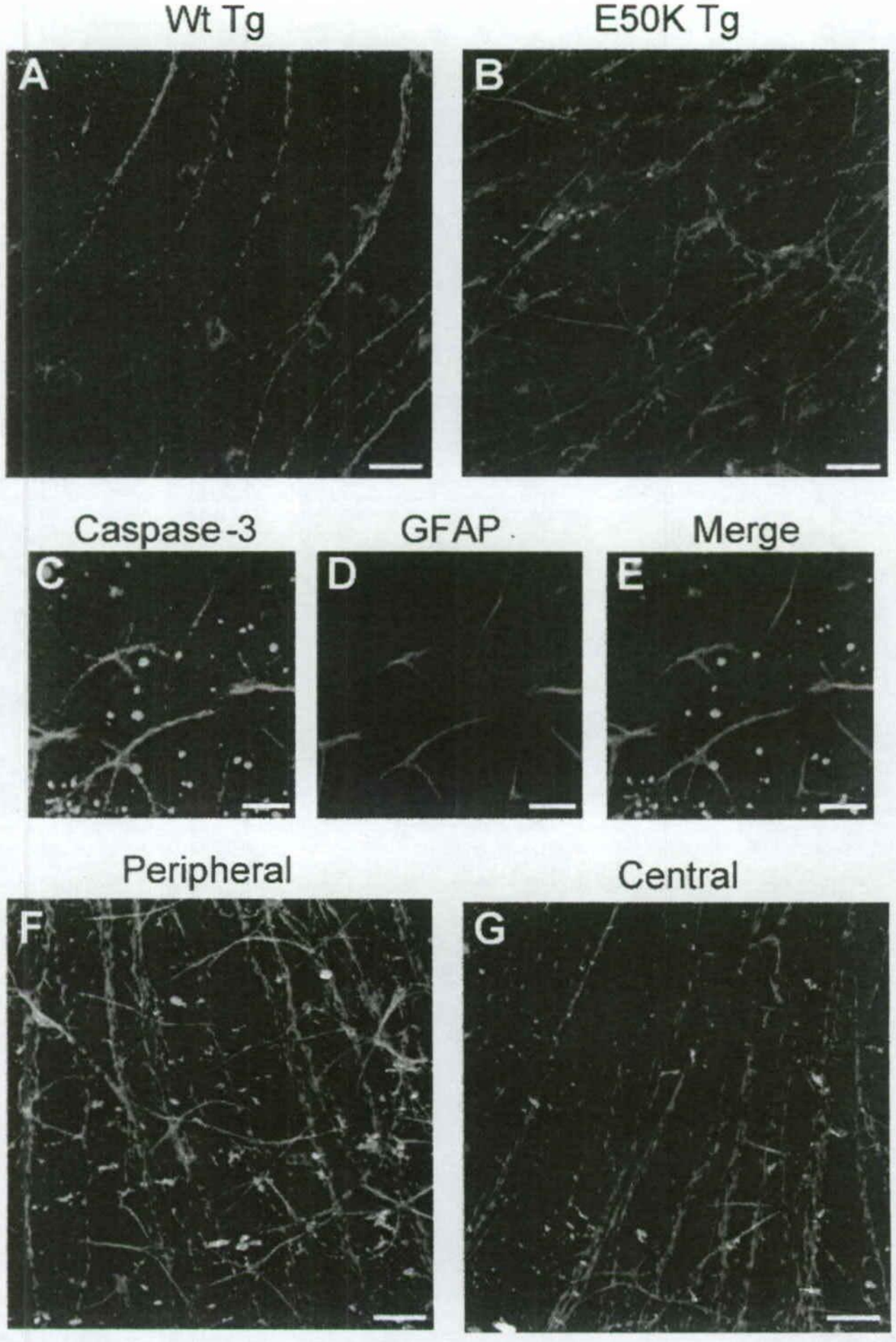
A



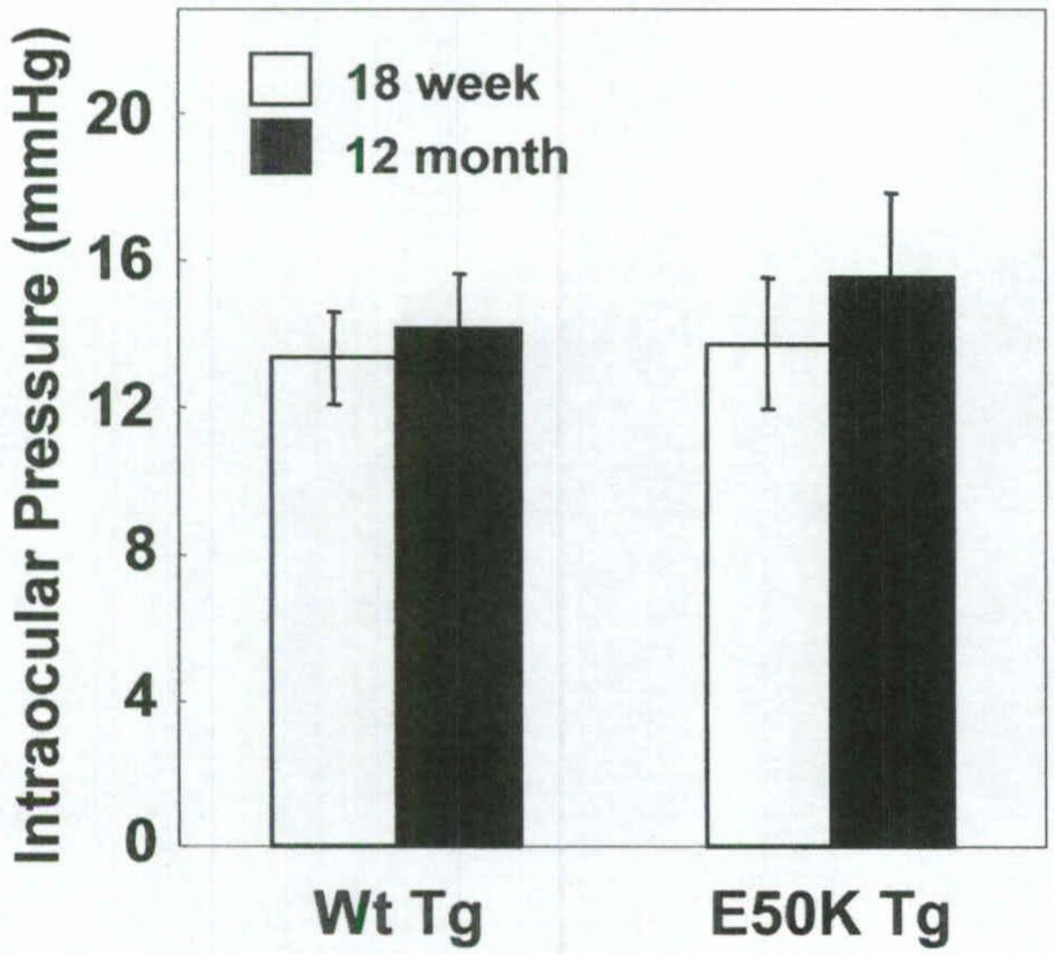
B



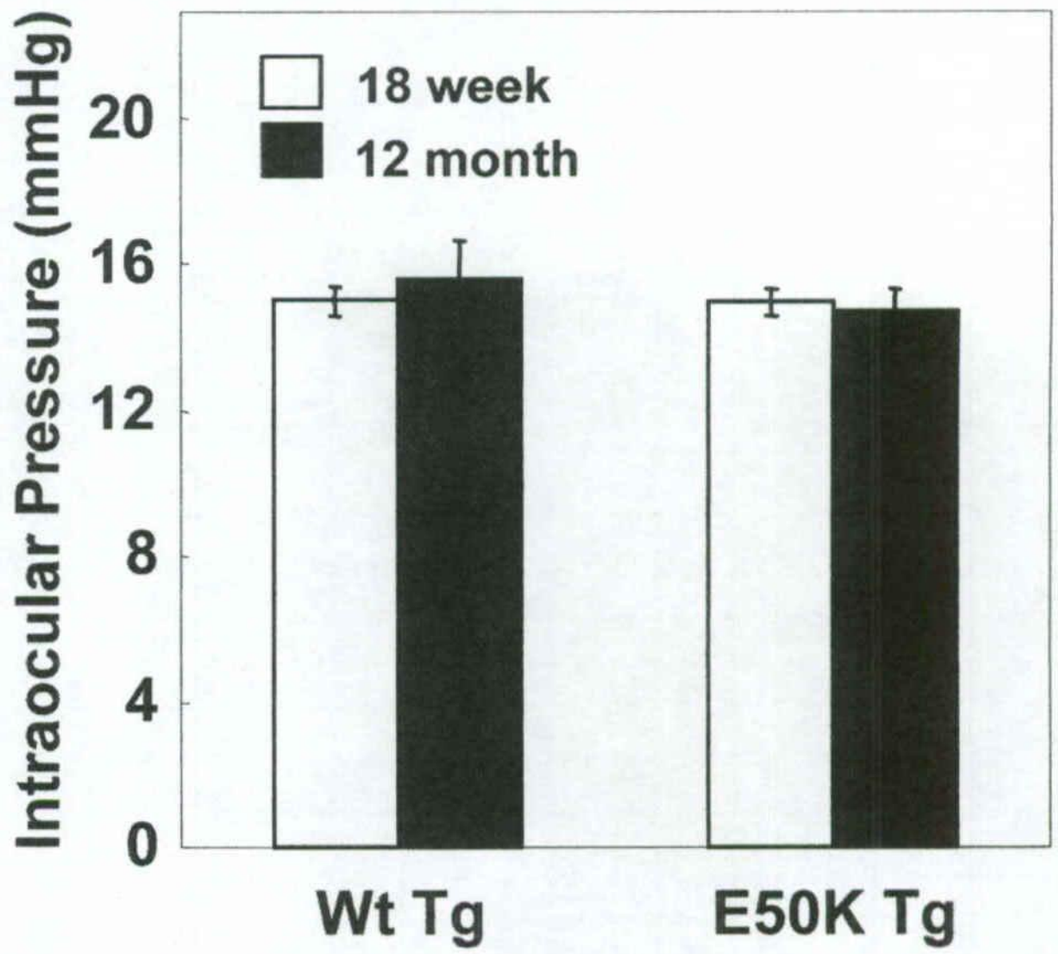

A
normal
Wt Tg
E50K Tg
B
C


Wt Tg
E50K Tg
A
B
Caspase-3
GFAP
Merge
C
D
E
Peripheral
Central
F
G

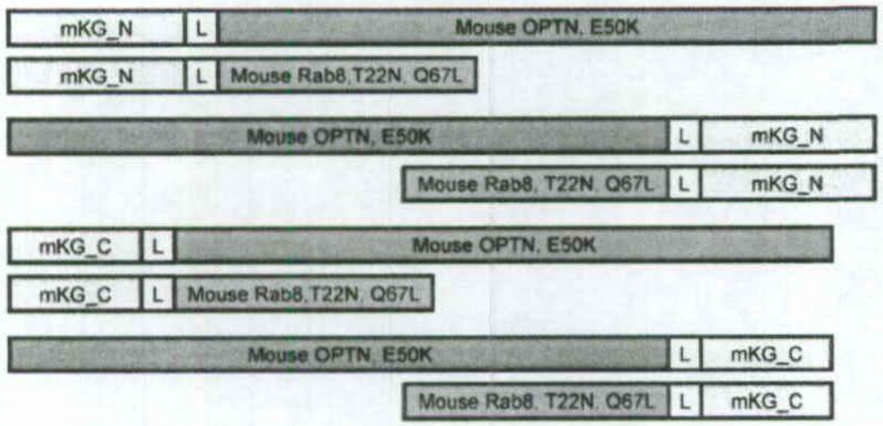
A


Impact Rebound Tonometer
18 week
12 month
Intraocular Pressure (mmHg)
0
4
8
12
16
20
Wt Tg
E50K Tg

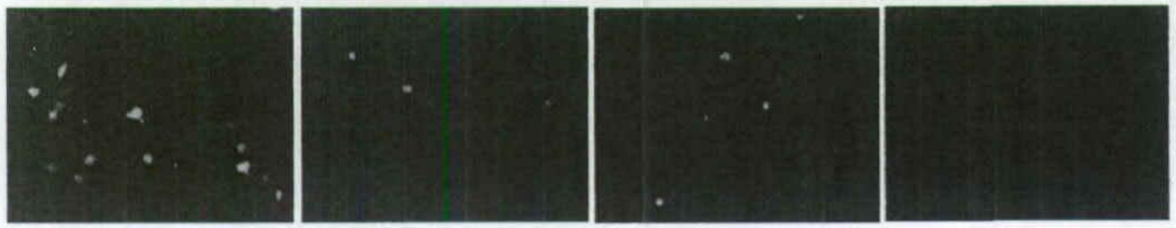
B

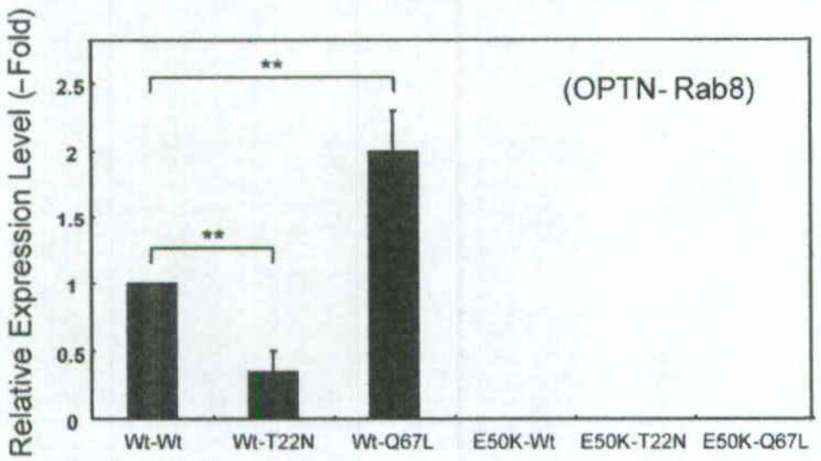

Optical Interferometry Tonometer
18 week
12 month
Intraocular Pressure (mmHg)
0
4
8
12
16
20
Wt Tg
E50K Tg

A

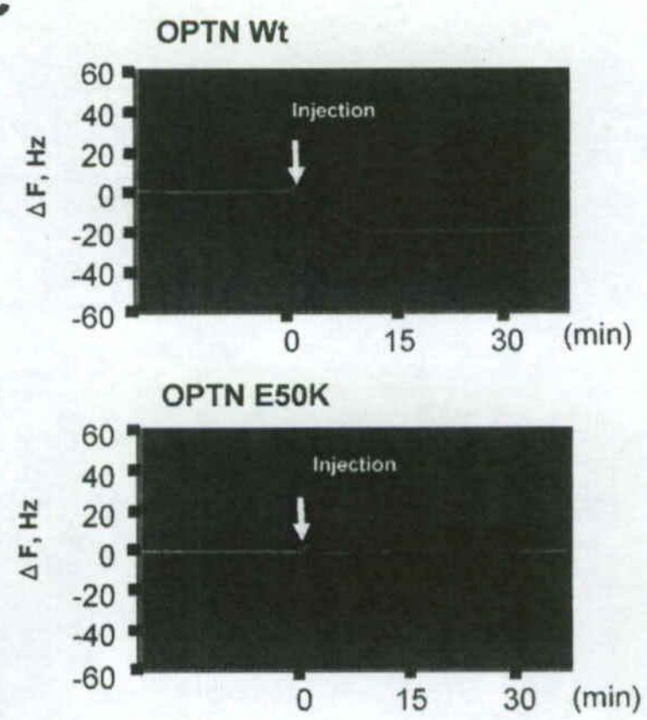

mKG_N
L
Mouse OPTN, E50K
mKG_N
L
Mouse Rab8,T22N, Q67L
Mouse OPTN, E50K
L
mKG_N
Mouse Rab8, T22N, Q67L
L
mKG_N
mKG_C
L
Mouse OPTN, E50K
mKG_C
L
Mouse Rab8,T22N, Q67L
Mouse OPTN, E50K
L
mKG_C
Mouse Rab8, T22N, Q67L
L
mKG_C

B

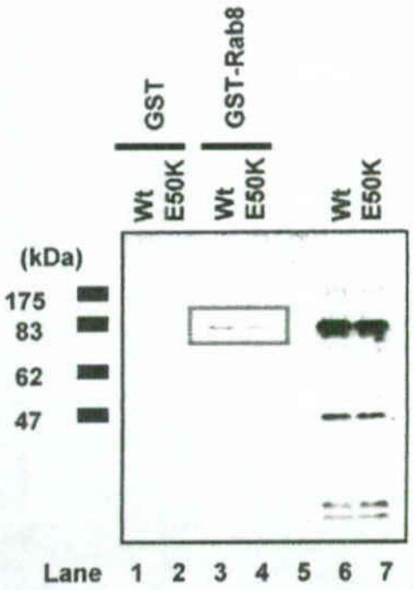

Positive Control
OPTN Wt-Rab8 Wt
OPTN Wt-Q67L
E50K-Q67L


Relative Expression Level (-Fold)
2.5
2
1.5
1
0.5
0
**
**
(OPTN- Rab8)
Wt-Wt
Wt-T22N
Wt-Q67L
E50K-Wt
E50K-T22N
E50K-Q67L

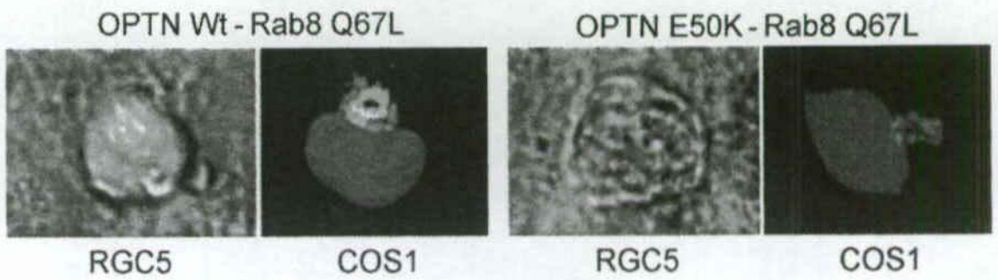
C

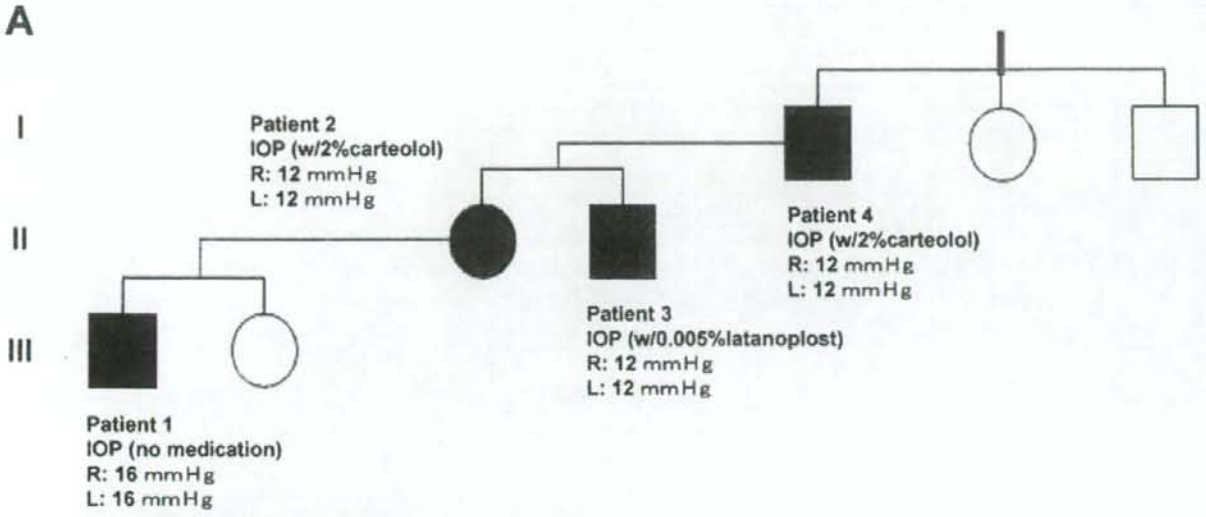

OPTN Wt
ΔF, Hz
60
40
20
0
-20
-40
-60
Injection
0
15
30
(min)
OPTN E50K
ΔF, Hz
60
40
20
0
-20
-40
-60
Injection
0
15
30
(min)

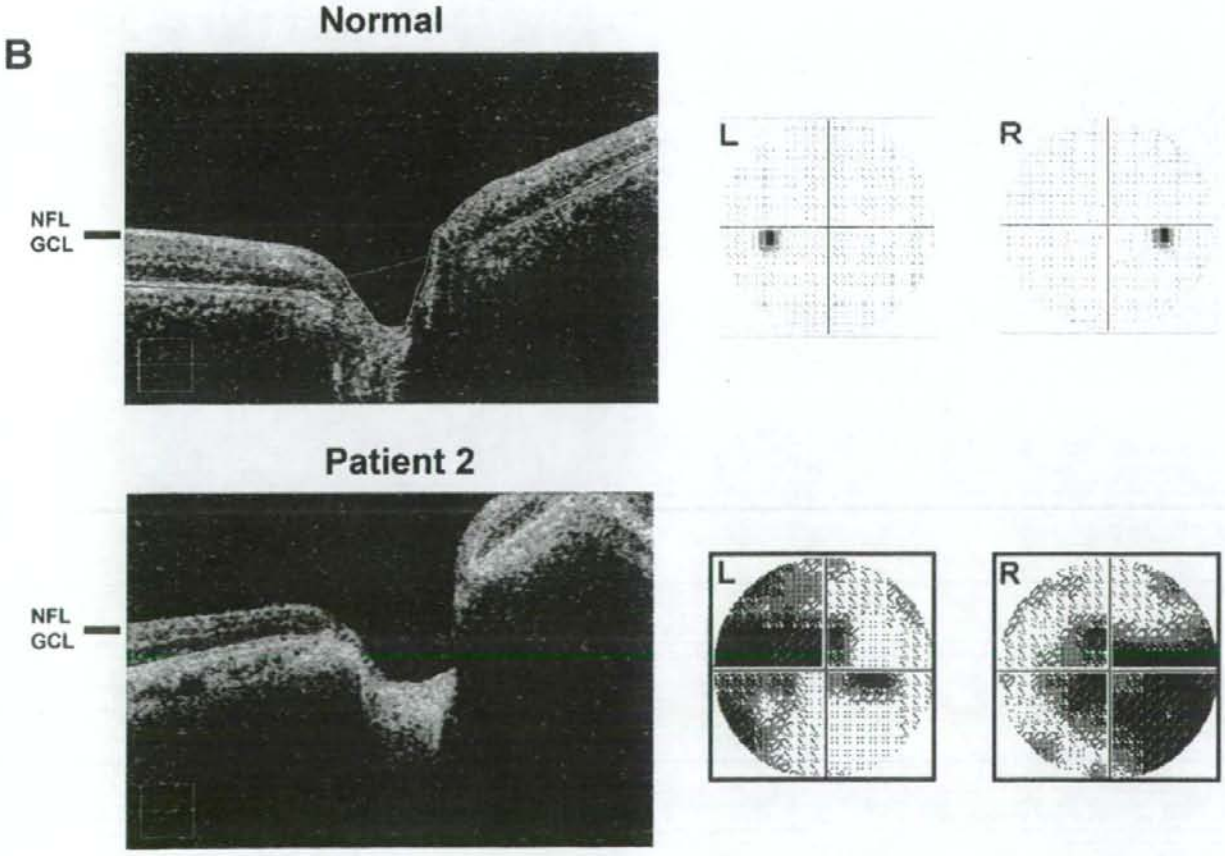
D


GST
GST-Rab8
Wt
E50K
Wt
E50K
Wt
E50K
(kDa)
175
83
62
47
Lane 1 2 3 4 5 6 7

E


OPTN Wt - Rab8 Q67L
OPTN E50K - Rab8 Q67L
RGC5
COS1
RGC5
COS1


A
I
II
III
Patient 2
IOP (w/2%carteolol)
R: 12 mmHg
L: 12 mmHg
Patient 4
IOP (w/2%carteolol)
R: 12 mmHg
L: 12 mmHg
Patient 3
IOP (w/0.005%latanoplost)
R: 12 mmHg
L: 12 mmHg
Patient 1
IOP (no medication)
R: 16 mmHg
L: 16 mmHg


B
Normal
NFL
GCL
L
R
Patient 2
NFL
GCL
L
R

High Resolution Genome-wide Association Study on Advanced Wet-type Age-Related Macular Degeneration

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Email: iwatatakeshi@kankakuki.go.jp

Word count:

Abstract

Age-related macular degeneration (AMD) is a multifactorial disease which affects mainly affects the central vision of the elderly population worldwide. The genetic contributions to the development of this disease have been extensively analyzed in various ethnic groups, and several susceptibility genes, e.g., complement factor H (*CFH*), *LOC387715*, and high temperature required A1 (*HTRA1*), have been identified. The latter two genes have been shown to be associate with all types of AMD, viz., geographic atrophy (dry-type), choroidal neovascularization (CNV, wet-type), and polypoidal choroidal vasculopathy (PCV-type). However, the mechanisms of how these genes participate in the onset of such different types of AMD are not well understood. Because many of the previous genome-wide association studies (GWAS) have includeddealt with both the dry-type and wet-type of AMD, the genetic factors participating in a particular type of AMD may have been overlooked.

A higher resolution GWAS using Affymetrix 500K array set was conducted on selected Japanese patients with only the advanced form of wet-type AMD, to identify the genes that which may lead to the severe wet-type AMD. A case-control study was performed on 100 advanced wet-type AMD and 200 controls. Only the *LOC387715/HTRA1* region on chromosome 10 was significantly associated with the

advanced wet-type AMD in the Japanese. The *CFH* region on chromosome 1 was not associated with the wet-type AMD. The *P* values of rs10490924, rs3750848 and rs2672587 in this region were 1.19×10^{-13}, 1.38×10^{-13}, and 8.02×10^{-9} respectively. We conclude that the *LOC387715/HTRA1* region is a unique locus that is strongly associated with the advanced wet-type AMD in Japanese.

Key words: age-related macular degeneration, genome wide association study, LOC387715, HTRA1

Introduction

AMD is a complex, multifactorial disease which is the main cause of irreversible decrease in vision in individuals over 65-years-of-age **[??worldwide??]** in western countries. The number of patients who are identified with AMD has been continuously increasing in Japan. AMD is a progressive disease with a wide spectrum of clinical findings that primarily affects the macular area of the retina.[1] The treatment of AMD has been primarily focused on the final stages of the disease, and consists of inhibiting the choroidal neovascularization (CNV) by antibodies against vascular endothelial growth factor (VEGF) or aptamer.[2] The results of recent studies have provided evidence that the risk factors for AMD are; genetics, behavioral, nutritional, and environmental factors.[1]

In Caucasians, early AMD is manifested by an increase in the number of large soft drusen, and the disease processes progresses to either the dry-type or wet-type AMD.[1] Recent studies have shown that both adaptive and innate immunological responses are activated around the retinal pigment epithelial (RPE) cells leaving traces of macrophages and activated complement factors in the drusen debris.[3]

In contrast to Caucasians with a high prevalence of the dry-type AMD with drusen, Japanese patients are predominantly affected with the wet-type AMD with CNVs and few

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