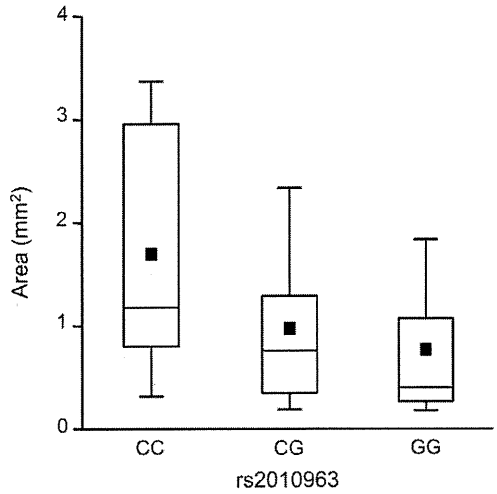

FIGURE 1. The area (mm²) of choroidal neovascularization among the three genotypes of rs2010963 in 83 patients. The area was significantly associated with the genotype ($P = 0.0047$).

genotypes of rs2010963 ($P = 0.54$, 0.98, and 0.69, respectively). To confirm the aforementioned association between rs2010963 and CNV size, we genotyped rs2010963 in an additional 76 patients with myopic CNV (20 male and 56 female). The genotype distribution of rs2010963 was significantly correlated with the CNV area ($P = 0.032$), while there was no significant difference in the axial length, age of patients, or male/female ratio among the three genotypes of rs2010963 ($P = 0.91$, 0.15, and 0.20, respectively). When these two cohorts were pooled for further evaluation of this association, the genotype distribution of rs2010963 was significantly correlated with the CNV area (Fig. 2, $P = 0.00078$).

DISCUSSION

In the present study, we found no association between VEGF gene polymorphisms and the occurrence of CNV in highly myopic eyes in Japanese patients, although rs2010963 was significantly associated with the size of CNV. To evaluate factors associated with CNV occurrence in highly myopic eyes, the age of the cohort is of critical importance. Therefore, when a younger cohort is used, some patients assigned to the group without CNV may eventually develop CNV, which can obscure potential differences between the two groups. Fernandez-Robredo et al. have evaluated the association of CFH Y402H and ARMS2 A69S polymorphisms with myopic CNV using 196 myopic patients who were aged ≥30 years.[27] We have previously evaluated the same association using 353 myopic patients who were ≥50 years of age,[28] and the present study consisted of 327 myopic patients who were aged ≥60 years. However, the association of VEGF gene polymorphism with CNV occurrence was not statistically significant. Furthermore, we evaluated the association using a cohort of patients older than 70 years, but statistical significance was still not found.

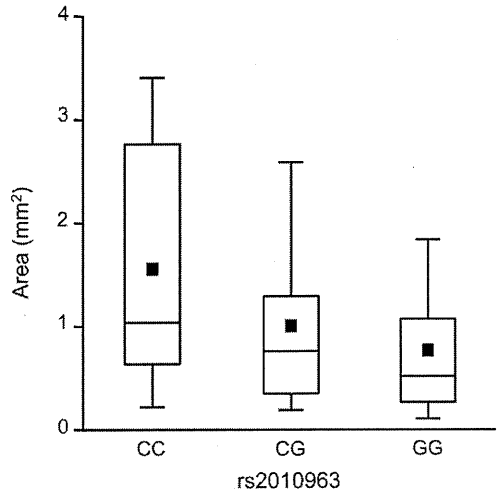

FIGURE 2. The area (mm²) of choroidal neovascularization among the three genotypes of rs2010963 in 159 patients. The area was significantly associated with the genotype ($P = 0.00078$).

Genetic associations with myopia have been investigated for several decades. Linkage studies have identified 18 possible loci for myopia (MYP1-18). Numerous candidate genes have been evaluated, and we have recently completed a GWAS study.[7] Furthermore, recent GWAS studies have revealed myopia susceptibility loci on chromosome 15, and we have successfully reproduced the association of these susceptibility loci with high myopia.[8-10] However, susceptibility genes for myopia have not been revealed; this makes it difficult to determine how to prevent myopia. Compared with the prevention of myopia, prevention and/or control of CNV occurrence and growth in highly myopic eyes might be a more practical approach. Since CNV is one of the most vision-threatening complications in highly myopic eyes, it is of great value to investigate the mechanism underlying CNV development in these eyes.

Although anti-VEGF treatments have been developed for the management of neovascular AMD, they are also substantially effective in treating myopic CNV.[11-13] Considering the effectiveness of these anti-VEGF treatments, we had hypothesized that VEGF is associated with the occurrence of CNV in highly myopic eyes. The present study, however, suggests that VEGF gene variations do not affect the occurrence of CNV in these eyes. In contrast with CNV occurrence, VEGF gene polymorphism rs2010963 was significantly associated with CNV size. Thus, it appears that VEGF contributes to CNV growth rather than CNV occurrence in highly myopic eyes. Experimental studies have shown that inhibition of VEGF leads to smaller CNV in laser-induced CNV models.[29-31] However, inhibition of VEGF does not always completely suppress CNV occurrence after laser photocoagulation to disrupt Bruch's membrane. This evidence suggests that VEGF only affects CNV size/growth, and other factors are responsible for triggering CNV occurrence, partly by interacting with Bruch's membrane.

The size of CNV is critical for visual prognosis in highly myopic eyes. Smaller CNVs can lead to smaller scotomas and spare the visual functions of the surrounding retina. Furthermore, very small CNVs can disappear completely after treatment.[6] Our findings suggest that development of larger CNVs in highly myopic eyes can be prevented by targeting VEGF, while prevention of CNV occurrence might be accomplished by targeting other factors.

Watson et al. reported that the amount of lipopolysaccharide-induced VEGF production from peripheral blood mononuclear cells (PBMNCs) is highest in individuals with a GG genotype of rs2010963, intermediate with a CG genotype, and lowest with a CC genotype.[17] In contrast to the findings of this study, we discovered that the size of CNV was largest in patients with a CC genotype, intermediate with a CG genotype, and smallest with a GG genotype. Considering that VEGF is a pro-angiogenic factor, these two findings seem contradictory. However, an evaluation of PBMNC function in in-vitro studies

does not always reflect their function in in-vivo situations. Furthermore, PBMNCs include several cell types such as lymphocytes, monocytes, and macrophages, and we have performed in vivo experiments that show that PBMNCs induce endothelium apoptosis[32] and that lymphocytes are negative regulators of pathological neovascularization, while monocytes are positive regulators in an ischemic retinopathy model.[33] Further studies are required to evaluate the roles of VEGF produced individually by monocytes or lymphocytes during myopic CNV development. In addition to VEGF produced from PBMNCs, VEGF produced from the RPE could also affect the growth of CNV in highly myopic eyes. Although we cannot evaluate the VEGF-producing ability of the RPE in an in-vivo situation, elucidation of the roles of the RPE in myopic CNV development might lead to better control of CNV size. It is also important to consider that VEGF can have several isoforms with different properties; we have demonstrated that VEGF165 is associated with pathological neovascularization, while VEGF121 is associated with physiological neovascularization.[33] Furthermore, recent studies have shown that some isoforms of VEGF are anti-angiogenic.[34] Additional studies on the role of different VEGF isoforms in myopic CNV development may lead to prevention of larger CNV secondary to high myopia.

Limitations of the present study include the age of the cohort and the small sample size. Although we used a cohort older than 60 years of age and performed a subanalysis using samples with patients older than 70 years, some participants included in the group without CNV might develop CNV in the future. Furthermore, our study is retrospective in nature, and the associations discovered herein need to be evaluated in prospective studies.

In conclusion, we have shown that VEGF gene polymorphisms have no association with the occurrence of CNV in highly myopic eyes in Japanese individuals; however, VEGF rs2010963 affects the size of CNV. Treatments that target VEGF may prevent large CNV formation in highly myopic eyes and help achieve better visual prognosis. To prevent CNV occurrence, further studies are needed to clarify the mechanism and/or background causes of CNV occurrence in highly myopic eyes.

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Nonimmunoglobulin target loci of activation-induced cytidine deaminase (AID) share unique features with immunoglobulin genes

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Activation-induced cytidine deaminase (AID) is required for both somatic hypermutation and class-switch recombination in activated B cells. AID is also known to target nonimmunoglobulin genes and introduce mutations or chromosomal translocations, eventually causing tumors. To identify as-yet-unknown AID targets, we screened early AID-induced DNA breaks by using two independent genome-wide approaches. Along with known AID targets, this screen identified a set of unique genes (*SNHG3, MALAT1, BCL7A,* and *CUX1*) and confirmed that these loci accumulated mutations as frequently as *Ig* locus after AID activation. Moreover, these genes share three important characteristics with the Ig gene: translocations in tumors, repetitive sequences, and the epigenetic modification of chromatin by H3K4 trimethylation in the vicinity of cleavage sites.

deep sequencing | end labeling by biotin oligonucleotide | microarray

Activation-induced cytidine deaminase (AID) is expressed in germinal center (GC) B cells upon antigen stimulation and is essential for two types of genetic alteration in the Ig gene: class switch recombination (CSR) and somatic hypermutation (SHM), which provide the genetic basis for antibody memory (1, 2). CSR produces antibodies with different effector functions by recombination at Ig heavy chain (H) switch (S) regions, so that the μ-chain constant (Cμ) region is replaced by a downstream C_H region. SHM introduces nontemplated point mutations in the rearranged variable (V) region genes, resulting in incremented antigen receptor affinity after clonal selection (3, 4).

Functional studies on AID mutants have shown that distinct AID domains are required for SHM and CSR, although AID has a single catalytic center (cytidine deaminase motif) in the middle of the molecule. Deletions and alterations in the N-terminal region affect both the CSR and SHM activities (5). However, AID C-terminal mutants almost completely lose CSR activity but retain or even increase SHM activity (6, 7). Although C-terminally truncated AID mutants cleave both V and S regions and induce enhanced c-myc-IgH translocations, they cannot mediate CSR, suggesting that the C-terminal domain is not required for DNA cleavage but is required to correctly pair cleaved ends (8).

The DNA cleavage of targets in CSR and SHM (the S region and V region, respectively) requires their transcription (9–12). Indeed, AID-induced mutations (SHM) are generally detected in a region within 2 kb downstream of the transcription start site (TSS) (13, 14). Transcription appears to play two roles in the targeting of cleavage sites. First, transcription is associated with the epigenetic marking of the target locus, particularly by H3K4 trimethylation (H3K4me3). The histone chaperone complex FACT is required to regulate H3K4me3 in the target S region, and FACT knockdown abolishes H3K4me3 and DNA cleavage in this region (15). Second, transcription is probably required to induce non-B structures in highly repetitive sequences such as S regions (16–18), due to excessive negative supercoiling induced immediately downstream of transcription. V regions have also been shown to form stem-loop structures under these conditions (19, 20). Non-B structure involvement has recently been reported in transcription-associated mutations in repetitive sequences such as the dinucleotide repeat hot spots or triplet repeat expansion/contractions causing Huntington's disease (17, 21, 22).

AID-dependent DNA cleavage is, in general, specific to the Ig locus. However, a number of reports have shown that AID can induce DNA cleavage in non-Ig loci. AID non-Ig targets were first demonstrated by studies on AID transgenic mice that produce numerous T lymphomas, in which vast numbers of mutations accumulate in the genes encoding the T-cell receptor, CD4, CD5, c-myc, and PIM1 (23, 24). This finding was followed by the observations that AID deficiency abolishes c-myc-Ig translocation and reduces the incidence of plasmacytoma (25, 26). AID expression is specific to activated B cells under normal conditions. However, AID expression has also been found in non-B cells, especially in cells stimulated by infection with pathogens such as human T-cell leukemia virus type 1 (HTLV1), hepatitis C virus (HCV), Epstein–Barr (EB) virus, and *Helicobacter pylori* (27–30). Based on these observations, AID is postulated to induce tumorigenesis, especially in B lymphomas and leukemias—and AID is expressed in many GC-derived human B-cell lymphomas (31–33). The prognosis of acute lymphocytic leukemia (ALL) and chronic myeloid leukemia (CML) is linked with AID expression (34, 35). It is therefore important to determine which non-Ig genes can be targeted by AID, and what features, if any, they share with Ig genes.

Several approaches have been used to explore AID non-Ig target genes in B cells. Candidate approaches involving the direct sequencing of proto-oncogenes, genes involved in translocations, or genes transcribed in normal GC B cells have shown that AID mutates several non-Ig genes, including *BCL6*, *MYC*, *PIM1*, and *PAX5* (24, 32, 36, 37). More recently, several efforts have been made to identify AID targets in a whole genome. These approaches have used chromatin immunoprecipitation (ChIP) of CSR-related proteins in combination with genome-wide tiling microarrays (ChIP-chip) or deep sequencing (ChIP-seq) on the assumption that proteins involved in CSR bind to AID targets. RPA, Nbs1, AID itself, and Spt5 have been used as marking proteins in this type of study (38–40). However, these approaches did not necessarily show that all of the protein-bound targets are cleaved or mutated by AID. There are indications that some genes identified by such approaches are not tran-

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The authors declare no conflict of interest.

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scribed (39). Therefore, it is important to reexamine non-Ig AID target genes by using a different strategy.

Here, we report four AID targets, identified by a combination of unique techniques. After directly labeling the DNA breakage ends from AID-induced cleavage with a biotinylated linker, we isolated the labeled fragments with streptavidin beads and analyzed them by a combination of promoter arrays and genome-wide sequencing. The candidates identified were then confirmed by quantitative PCR (qPCR) and the actual demonstration of mutations. With these methods, we identified at least four previously unknown AID targets—*SNHG3, MALAT1, BCL7A,* and *CUX1*. We found that these targets share important characteristics with Ig genes, namely, repetitive sequences that can form non-B structures upon efficient transcription, and the accumulation of H3K4me3 histone modifications on the chromatin.

Results

AID-Induced DNA Cleavage Detected by Labeling DNA Break Ends with a Biotinylated Linker. To detect genome-wide AID-induced DNA breaks, we used a modified in situ DNA end-labeling technique as described (8, 41) in BL2 cells, a Burkitt's lymphoma cell line that serves as an in vitro model for studying the SHM mechanism (31, 42, 43). We used the BL2 clone BL2-ΔC-AIDER, which expresses JP8Bdel, an AID mutant lacking the C-terminal 16 residues, fused with the hormone-binding domain of the estrogen receptor (ER) (JP8Bdel-ER). Tamoxifen (4-OHT) treatment induces DNA breakage in the Sμ and Sα regions but not in the Sγ region of JP8Bdel-ER-expressing CH12 cells, which switch almost exclusively from IgM to IgA (8).

BL2-ΔC-AIDER cells were treated with 4-OHT only for 3 h to minimize cell death and DNA break ends were labeled with a biotinylated linker, and the break-enriched biotinylated DNA was used as a PCR template (Fig. 1*A*). In agreement with previous reports (8, 42), we detected DNA breakage in the 5′ Sμ region of the IgH locus only in 4-OHT–treated cells. No breakage was detected in the *B2M* gene, which is expressed in BL2 cells but was shown not to accumulate mutations in activated B cells (Fig. 1*B*).

AID Targets Identified by Promoter Array and Whole Genome Sequencing. Because SHM is normally detected close to the TSS (13, 14), biotin linker-enriched DNA fragments were analyzed by a promoter array to identify unknown AID targets. Table S1 lists the genes whose signals increased after 3 h of 4-OHT treatment, compared with untreated samples with false discovery rate (FDR) values <0.3. We also looked for genes with increased signals after 4-OHT treatment that are known to be targets of chromosomal translocation or genes that had multiple breakage peaks, and we identified >50 genes, among which we found that *BCL7A* and *CUX1* are enriched in the original breakage-enriched library by qPCR (see below). We confirmed by RT-PCR and expression array that *SNHG3*, *MALAT1*, *NIN*, *C9orf72*, *CFLAR*, *SNX25*, *BCL7A,* and *CUX1* were transcribed in BL2 cells (Table S1). Fig. S1 shows the peak signals in a 10-kb segment surrounding the breakage area of *SNHG3, MALAT1, BCL7A,* and *CUX1*. We could not map the breakage in the Ig locus because of the absence of array probes in this region.

Because the promoter array does not detect DNA fragments outside of regions containing probes, we further analyzed the breakage-enriched DNA by direct sequencing of the biotin linker-enriched library. DNA breakage sites in both control and 4-OHT–treated libraries were identified by aligning sequenced tags to the genome, and significantly enriched regions were identified by comparing the local breakage density (*SI Materials and Methods*). Regions were identified in the genes listed in Table S2. Interestingly, *SNHG3* and *MALAT1*, which were identified by the promoter array, appear at the top of the list in the genome-wide sequencing as well.

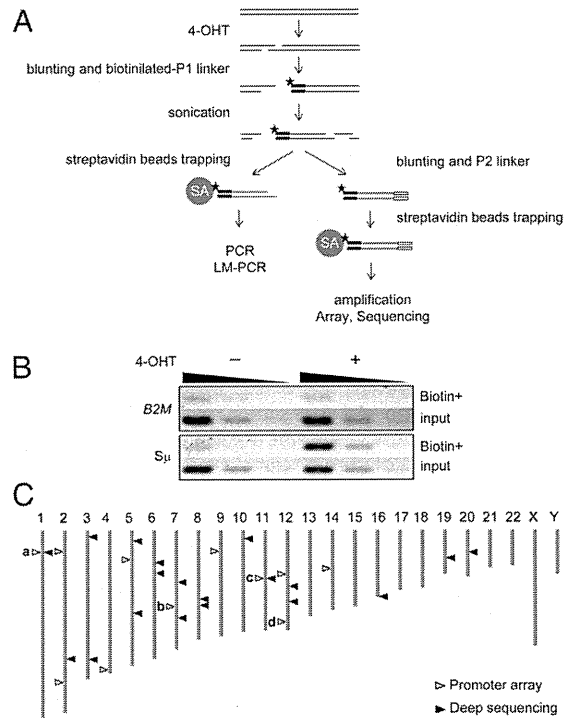

Fig. 1. (*A*) Schematic of the labeling technique. 4-OHT is added to activate AID, and DNA break ends are labeled in situ by biotinylated linker ligation. After genomic DNA is extracted and sonicated, biotinylated fragments are captured by streptavidin beads and used for PCR, array, or sequencing. (*B*) Detection of DNA breaks by PCR. BL2-ΔC-AIDER cells were treated with or without 4-OHT for 3 h, and the break ends were labeled. PCR of Sμ and *B2M* was performed with biotin-labeled DNA or input DNA by using fivefold serially diluted templates. (*C*) Chromosomal distribution of AID targets. a, *SNHG3*; b, *CUX1*; c, *MALAT1*; d, *BCL7A*. White arrowhead, promoter array (FDR < 0.3 plus *BCL7A* and *CUX1*); black arrowhead, whole genome sequencing (FDR < 0.01 and/or remarkable numbers of *P* value clusters).

Fig. 1*C* shows the chromosomal distribution of AID target candidates identified by promoter array or whole-genome sequencing. Breakage seemed to be distributed through the genome without any apparent bias. Surprisingly, of the 29 candidates identified by whole-genome sequencing with strict statistical parameters, only two matched candidates obtained from the promoter array. This discrepancy might be explained in part because most of the breakage-rich regions detected by whole genome sequencing are located in regions that do not contain promoter array probes.

Results may also be limited because of possible bias by PCR amplification of the primary library for microarray and whole-genome sequencing, which could affect the relative genome coverage. To avoid this bias, we relied on the original library and confirmed all candidates by qPCR.

qPCR Analyses of Linker Libraries. To confirm the AID-induced breakage candidates detected by the promoter array and whole-genome sequencing, we used qPCR assays with gene-specific primers to amplify the vicinity of the identified breakage regions in biotin linker-enriched DNA from cells treated with 4-OHT for 3 h (Fig. 2). We examined whether candidate genes were enriched in the 4-OHT–treated DNA library compared with the nontreated library. Among the 29 candidates identified by whole-genome sequencing, only *SNHG3* and *MALAT1* were strongly enriched ($P < 0.0001$ and $P < 0.001$, respectively). Besides these, *BCL7A*, *CUX1,* and *CFLAR*, which were picked up only by the

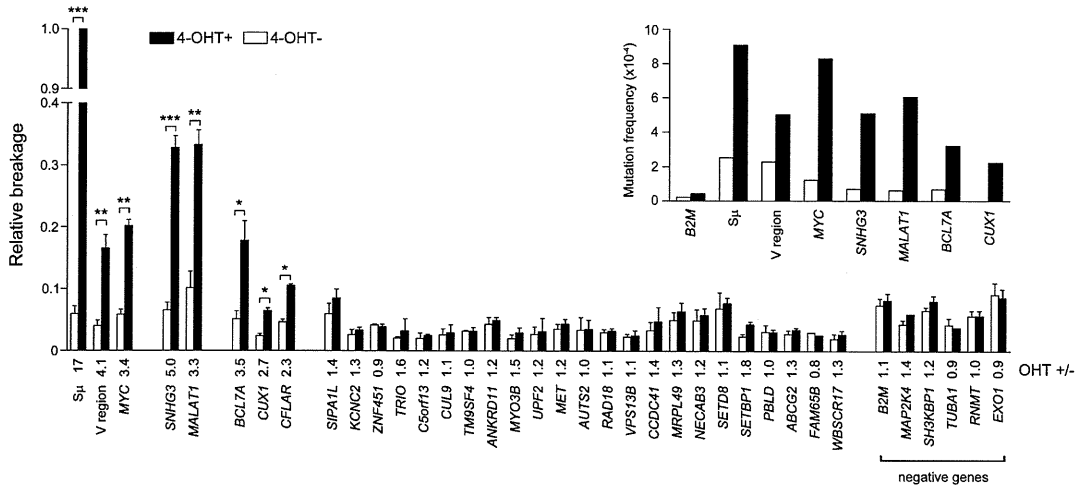

Fig. 2. qPCR measurement of DNA breaks. Break signals are presented relative to Sμ. SD values were derived from at least three independent experiments, and *P* values were calculated by a two-tailed *t* test. $*P < 0.01$, $**P < 0.001$, $***P < 0.0001$. Numbers below the *x* axis indicate the ratio between samples treated and not treated with 4-OHT. (*Inset*) Mutation analysis of genes with significantly increased break signals after AID activation. Cells were treated with or without 4-OHT for 24 h. Only unique mutations were counted. Detailed mutation profiles can be found in Fig. S2 and Table S3.

promoter array, also showed significant enrichment ($P < 0.01$) in the 4-OHT–treated library.

We also confirmed that the Sμ and V regions in BL2 cells were cleaved, because they were enriched in the 4-OHT–treated library. Although *MYC*, which is translocated in an AID-dependent manner in human Burkitt's lymphoma (44), was not identified by either promoter array or whole-genome sequencing, qPCR of the 4-OHT–treated samples clearly revealed *MYC* gene enrichment (Fig. 2). The difference in cleavage detection between the direct candidate qPCR and genome-wide arrays and sequencing suggests that the amplification step required for microarray and whole-genome sequencing methods may introduce bias, either for or against many genes. In the case of sequencing, this bias can lead to low mapping coverage of certain regions, hampering efforts to identify significant enrichment. Therefore, we cannot exclude genes that were not identified by the present methods from being AID targets.

AID Targets Accumulate Somatic Mutations near Cleavage Sites. To test whether the newly identified target genes are mutated upon AID activation, we treated BL2-ΔC-AIDER cells with 4-OHT for 24 h and sequenced regions of ≈600 bp around each area with abundant breakage (Fig. S2 and Table S3). Mutations increased in all of the qPCR-confirmed AID target genes after 4-OHT treatment (Fig. 2, *Inset*), with mutation frequencies ranging from 6.1×10^{-4} for *MALAT1* to 2.2×10^{-4} for *CUX*. These frequencies are comparable to those of the V region (5.0×10^{-4}), the Sμ region (9.1×10^{-4}), and the *MYC* gene (8.3×10^{-4}), and are far higher than that of the control *B2M* gene (4.3×10^{-5}). We also detected mutations in the *CFLAR* gene; however, the mutation frequency (9.2×10^{-5}) was not as high as other AID target genes, although mutations increased significantly in 4-OHT–treated sample ($P = 0.004$) (Table S3).

To compare the distribution profiles of mutated bases and AID-induced DNA breaks in the biotin linker-enriched DNA, we mapped the linker positions by performing ligation-mediated (LM)-PCR with the linker primer and gene-specific primers. These PCR fragments were subsequently cloned and sequenced. Break ends identified by the linker were plotted, together with mutation positions (Fig. 3 and Fig. S2). The results clearly showed that the DNA cleavage marks (biotin linker) were closely associated with mutations, indicating that the DNA cleavage sites identified are functionally relevant to SHM by AID. We used RT-PCR and expression arrays to confirm that the regions

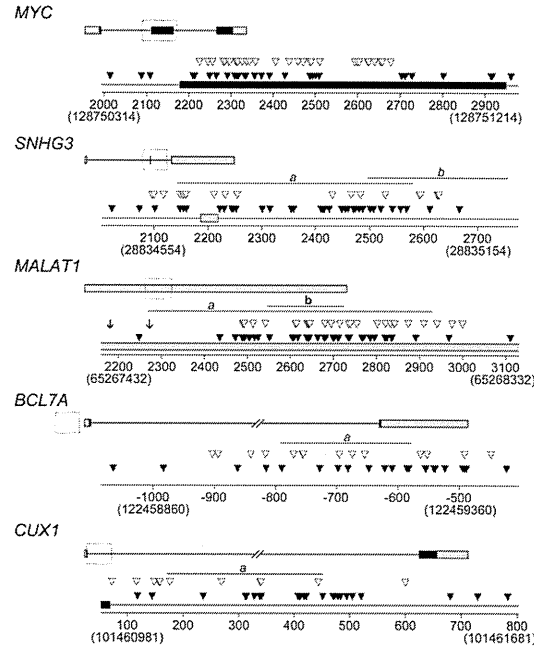

Fig. 3. Somatic mutations and breakpoint distribution in AID target loci. Mutations (open triangles) and breakpoints (filled triangles) detected by LM-PCR (Fig. S2) were plotted on the respective genomic sequences. The top scheme represents exons (rectangles) and introns (bars). Genomic loci are shown in untranslated and translated sequences (gray and black boxes, respectively). The horizontal lines *a* and *b* represent breakage regions identified by promoter array and sequencing, respectively. Regions outlined by dotted boxes are shown in more detail below each genomic locus. For the *MALAT1* locus, the translocation breakpoints reported by Davis et al. (45) are represented by arrows. *x* axis numbers indicate base positions according to RefSeq: NM_002467 (*MYC*), NR_002909 (*SNHG3*), NR_002819 (*MALAT1*), NM_020993 (*BCL7A*), and NM_181552 (*CUX1*). Numbers in parentheses indicate the corresponding base position according to hg19 assembly.

where DNA cleavage and mutations were identified are transcribed (Tables S1 and S2).

Repetitive Sequences Surround the Breakage Regions of Unique Targets. We next examined common features among the AID targets. Although SHM has been reported to prefer the RGYW-WRCY motif (46), we could not find any enrichment of this motif among the break sites in the newly identified targets. It was recently reported that mutations are introduced in regions with sequences prone to forming non-B DNA structure, including tandem repeats, palindromes, and inverted repeats (17, 18). The S region, *MYC,* and V region genes contain sequences prone to forming non-B structure (19, 20, 47, 48). We used REPFIND, a program that identifies clustered, nonrandom short repeats in a given nucleotide sequence, to search the vicinity of identified breakage regions for sequences prone to forming non-B structure. For each repeat cluster, a *P* value is calculated indicating the probability of finding such a repeat cluster randomly (a *P* value of 1×10^{-5} means that such a concentration of that particular repeat occurs an average of once in 100,000 bp by chance) (49). Curiously, we found that various types of repeat sequences cluster in the vicinity of cleaved sites in the newly identified AID target genes. In the *MALAT1* locus, the region within 2 kb surrounding the breakage peaks was rich in clustered short repeat motifs such as GAAG, GCC, GAA, CCG, AAG, GAAGA, and TTAA (Fig. 4). Repeat clusters were also found near the cleavage sites of the *SNHG3*, *BCL7A,* and *CUX1* loci. (Fig. S3). In all cases, the probability of the appearance of these repeats was far below random ($P < 1 \times 10^{-8}$).

H3K4me3 at Cleavage Sites. It was recently shown that S region transcription alone is not sufficient for CSR; specific histone posttranslational modification marks, especially H3K4me3, are required. H3K4me3 depletion strongly inhibits CSR and DNA cleavage in the Sμ and Sα regions (15). We thus asked whether the V region and the newly identified AID targets also carry H3K4me3 marks around the cleavage regions. ChIP analysis showed that both the V region and *MALAT1* locus were abundantly marked by H3K4me3 (Fig. 5). Furthermore, the H3K4me3 distribution profiles corresponded well to the somatic mutation distribution in the rearranged V region and to the breakage signal

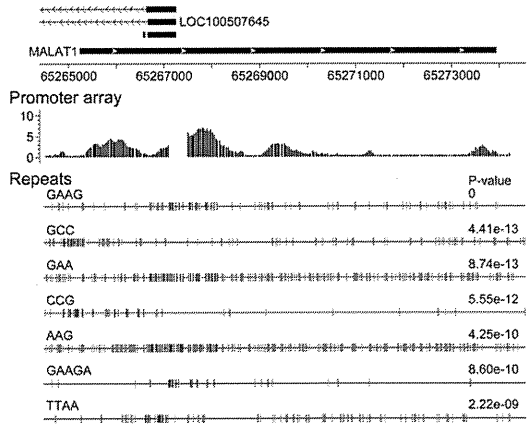

Fig. 4. Repeat sequences surrounding the breakage region in the *MALAT1* gene. (*Top*) Representation of a 10-kb segment surrounding the *MALAT1* locus. *x* axis numbers represent base positions according to hg19 assembly. (*Middle*) Breakage signal distribution detected by promoter array. Regions without bars do not have array probes. (*Bottom*) REPFIND analysis showing significant repeat clusters in the *MALAT1* locus. Motifs depicted as small, colored, vertical bars indicate the cluster with the most significant *P* value; individual repeats are separated by different colors.

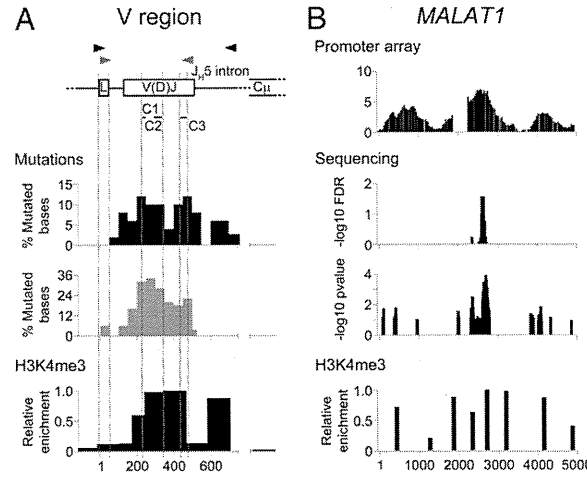

Fig. 5. H3K4me3 distribution in the IgH V region and in the *MALAT1* gene. (*A Top*: Representation of the rearranged IgH V region of BL2 cells. Black and gray arrowheads represent the position of primers used for the mutation analysis shown in *Bottom* (graphs in black and gray, respectively). L, leader; C1, CDR1; C2, CDR2; C3, CDR3. (*A Middle*) Somatic mutation distribution, represented as the percentage of mutated bases per 50 bp sequenced. Graph in black: mutations from Fig. 2, *Inset*. Graph in gray: mutations reported by Denepoux et al. (50). (*Bottom*) ChIP assay using an anti-H3K4me3 antibody. *x* axis numbers indicate the nucleotide position relative to the first V-gene ATG. (*B*) *MALAT1* locus. From top to bottom: Breakage signal distribution detected by promoter array (regions without bars do not have array probes); FDR regions by sequencing; *P* value peaks by sequencing; ChIP assay using an H3K4me3 antibody. *x* axis numbers indicate base positions according to RefSeq NR_002819.

distribution observed by both the promoter array and whole genome sequencing in *MALAT1* (Fig. 5 *A* and *B*). Mutations identified in *MALAT1* overlapped with DNA cleavage signals and H3K4me3 marks (Figs. 3 and 5*B*). We examined the H3K4me3 pattern of other AID targets by using publicly available ENCODE ChIP-seq data for the B-lymphoblastoid cell line GM12878 (51). As expected, all of them, except for *BCL7A*, were highly abundant in H3K4me3 marks overlapping nicely with cleavage sites (Fig. S4). H3K4me3 might be absent at the *BCL7A* locus in GM12878 cells because it is an inducible gene expressed in BL2 cells, but not in the GM12878 cell line (52). We thus conclude that the newly identified AID targets share both *cis* and *trans* marks for AID targeting—non-B structure and H3K4me3, respectively (15, 16).

Discussion

Identified AID Targets Accumulate High-Frequency Mutations. We explored AID targets by combining three different strategies: promoter array, whole genome sequencing, and candidate qPCR in a library containing biotinylated linker-labeled cleaved ends. With these strong criteria, we were able to identify four unique AID targets: *SNHG3, MALAT1, BCL7A,* and *CUX1*. All of these candidates were further confirmed to accumulate mutations. These candidates are thus strong AID cleavage targets; however, these genes represent only very efficient AID targets. The use of the biotinylated linker, which efficiently identifies double-strand breakage with close, staggered nicks on opposite strands, may not detect scattered nicks efficiently, and this may limit identification to targets that are efficiently and specifically cleaved within 3 h of AID activation.

Some well-described SHM target genes, including *MYC, BCL6, PAX5, RHOH,* and *PIM1,* were not detected by either the promoter array or whole genome sequencing. We used qPCR to test whether these genes were enriched in the biotin-labeled

DNA library, but only *MYC* was enriched in the 4-OHT–treated sample (Fig. 2). These genes have been found to be mutated in memory and GC B cells as well as lymphoma cells (24, 32, 36, 37), cells that are expected to be chronically exposed to AID. In addition, the mutation accumulation in tumor cells depends on selection. In contrast, in our study, we exposed BL2 cells to a short treatment (3 h) of 4-OHT, to increase the chance of detecting only efficiently targeted loci. In fact, none of the genes above mentioned mutated more than 1/20th of the 3′ J_H locus even in 6-mo-old Peyer's patch B cells (36).

The unique AID targets accumulate mutations at comparable frequencies with the *Ig* and *MYC* genes. We found that the mutation and cleavage sites are located in similar areas. The results indicate that the cleavage and mutation sites are linked, but not necessarily identical. This observation is consistent with the prediction that SHM is incorporated during the repair phase by error-prone polymerases (53). We confirmed that all of the newly identified AID targets were highly transcribed in BL2 cells. Although the breakage signal detected at the *BCL7A* locus was ≈800 bp upstream of the TSS, we detected both sense and antisense transcripts in this region.

Unique AID Targets also Translocate. Furthermore, it is important to stress that all of these unique candidates have been shown to be the targets of chromosomal translocation in neoplastic cells as shown for the *Ig* locus and *MYC* gene. MALAT1 is overexpressed in several cancers and was reported to be involved in regulating alternative splicing (54). The *MALAT1* locus has been found to harbor chromosomal translocation breakpoints associated with cancer (45, 55) and, interestingly, two reported translocation breakpoints are close to or within the breakage region identified in the present study (Fig. 3). *SNHG3*, a host gene for small nucleolar RNAs (56), is also reported to be involved in translocation, and although the exact position of the translocation breakpoint has not been reported, we can speculate that it is located in the second intron of *SNHG3* because the detected fusion transcript joins the second exon of *SNHG3* with the exon of the 3′ partner gene (57). *BCL7A* and *CUX1* have also been reported to bear chromosomal translocations; however, these translocation breakpoints occur far from the breakage regions identified in this study (58, 59).

Abundant Repetitive Sequences in AID Targets. To identify common features of AID targets, we compared the *MYC*, *SNHG3*, *MALAT1*, *CUX1*, and *BCL7A* genes with the Ig gene locus (the V_H gene and the Sμ region). Sequence analysis identified abundant repetitive sequences surrounding the cleaved regions of AID targets. A typical example is *MALAT1* (Fig. 4): The GAAG, GCC, GAA, CCG, AAG, GAAGA, and TTAA repeats are highly abundant within 2 kb surrounding the break peaks, which also overlap with actual mutation sites. In the *SNHG3* locus, less frequent but longer repeats—GGATTACAG, TTTTTGTATTTT, ATTACAGGC, GCCTC, and TTTTTGTA—are clustered in the proximity of cleavage sites (Fig. S3*A*). *BCL7A* and *CUX1* have GC-rich repeats, such as CGCG, CCGCG, CCCG, and CGGCG (Fig. S2 *B* and *C*). The *MYC* gene, the V region, and the S region are already known to have repetitive sequences or inverted repeats that can form non-B structure when the target is actively transcribed and under an excessive negative superhelical condition (19, 20, 47, 48).

H3K4me3 Marks in AID Targets. Chromatin modifications are also involved in AID targeting. We showed that H3K4 methylation, specifically trimethylation, is critical for DNA cleavage in the S region (15), although Odegard et al. (60) showed that the H3K4 dimethylation (H3K4me2) pattern is similar among VJλ1, Cλ1, and Eλ3-1 and concluded that H3K4me2 is not correlated with SHM. Association of H3K4me3 with the MYC locus was also reported (38). Therefore, we tested whether H3K4me3 modification is also associated with the V region and the unique loci. SHM in V regions typically targets the whole coding V-region segment and extends to its 5′ and 3′ flanking regions. Mutation frequencies rise sharply ≈100 bp downstream of the TSS (at the middle of the leader intron), peak in V(D)J, and then gradually decrease after the immediate 3′ flanking region, becoming undetectable over a distance of ≈1 kb from the rearranged J (61). It is striking that the H3K4me3 profile follows the exact same tendency as SHM distribution in the V region (Fig. 5*A*). H3K4me3 is scarce in the leader exon and intron but present in the highly mutated portion of the V(D)J exon. We also observed that H3K4me3 distribution at the *MALAT1* locus corresponded well with the breakage signal distribution detected by both the promoter array and whole genome sequencing (Fig. 5*B* and Fig. S3*A*). The H3K4me3 pattern of other AID targets also overlaps with cleavage sites (Fig. S3 *B*–*D*). Strikingly, we observed a strong H3K4me3 peak in the 5′ region of the *CUX1* gene (Fig. S4*D*), which does not contain microarray probes. We confirmed that this region also accumulates mutations after 4-OHT treatment (Table S3). It would be interesting to check whether H3K4me3 depletion can decrease AID-induced breaks and mutations in the newly identified AID targets.

We thus conclude that all of these genes, *SNHG3, MALAT1, BCL7A,* and *CUX1*, share unique characteristics that are required for AID targeting: non-B structure as the *cis* element and the H3K4me3 histone modification as the *trans* mark.

Materials and Methods

Labeling of DNA Break Ends by a Biotinylated Linker. The biotin-labeled DNA break assay was performed as described (8) with slight modifications. After nuclear permeabilization, BL2 cells were washed with cold PBS and resuspended in 1× T4 DNA polymerase buffer. Blunting was performed by using T4 DNA Polymerase (Takara). After washing with cold PBS, 4 μL of T4 DNA Ligase (Takara) and 13.4 μL of an annealed biotinylated P1 linker were added, and the cells were incubated overnight at 16 °C. Genomic DNA was purified by phenol:chloroform extraction.

PCR, Real-Time PCR, and LM-PCR. Biotinylated genomic DNA (10 μg) was sonicated (Covaris) and incubated with 10 μL of M-270 Dynabeads (Invitrogen) for 15 min at room temperature. After washing, the beads were resuspended in 15 μL of TE buffer and used as a PCR template. PCR was initiated by denaturing for 5 min at 95 °C followed by 25 cycles (95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s) and a final extension at 72 °C for 5 min. SYBR Green Master Mix (Applied Biosystems) was used for real-time PCR.

For LM-PCR, we used a template of 1 μL of beads in a two-round PCR by using linker primer (P1-LM) and gene-specific primers. First-round PCR was initiated by nick translation (72 °C for 20 min), followed by denaturing (95 °C for 5 min), 25 cycles (95 °C for 15 s, 65 °C for 15 s, and 70 °C for 1 min), and a final extension (70 °C for 5 min). Second-round PCR included denaturing (95 °C for 5 min), 20 cycles (95 °C for 15 s, 65 °C for 15 s, and 70 °C for 1 min), and a final extension (70 °C for 7 min). The PCR fragments were purified, cloned with the pGEM-T Easy Vector System (Promega), and sequenced with the ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). Primers sequences are provided in Table S4–S7.

DNA Preparation for Microarray and SOLiD Sequencing. After sonication of biotin-labeled genomic DNA, sheared ends were blunted by adding T4 DNA polymerase for 30 min at room temperature. DNA was purified by using the PureLink PCR purification Kit (Invitrogen), P2-annealed linker was ligated overnight at 16 °C, DNA was incubated with Dynabeads as described above, and the beads were used for global amplification by following the SOLiD protocol (Applied Biosystems). A summary of general features of the sequenced libraries can be found in Fig. S5 and Table S8.

Accession Codes. Gene Expression Omnibus: microarray data, GSE32027; DNA Data Bank of Japan: sequencing data, DRA000450.

Other material and methods are provided in *SI Materials and Methods*.

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Significance of *C2/CFB* Variants in Age-Related Macular Degeneration and Polypoidal Choroidal Vasculopathy in a Japanese Population

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Purpose. To determine whether genetic variants in the complement component 2 and factor B gene (*C2/CFB*) locus are associated with the risk for typical age-related macular degeneration (AMD) or polypoidal choroidal vasculopathy (PCV) in a Japanese population.

Methods. Four single nucleotide polymorphisms (SNPs) were genotyped across the *C2/CFB* locus of patients with typical AMD (n = 455) or PCV (n = 581) and of 865 controls. Differences in the observed genotypic distribution between the case and control groups were tested by logistic regression analysis for age and sex adjustments. Significant associations were confirmed using a second control group of 336 cataract patients. A further model adjusting for age-related maculopathy susceptibility 2 (*ARMS2*) A69S, complement factor H (*CFH*) I62V, age, sex and smoking status was performed, to confirm their independent association from other covariates.

Results. *C2* rs547154 and *CFB* rs541862 were significantly associated with typical AMD and PCV in this Japanese sample ($P < 0.05$). These two SNPs were also significantly associated with typical AMD and PCV in evaluation of the second control cohort ($P < 0.05$). Furthermore, an independent association of *C2/CFB* variants was found for both typical AMD and PCV with age, sex, smoking, and genetic background of *ARMS2* A69S and *CFH* I62V (vs. typical AMD: $P = 0.0073$, odds ratio [OR] = 0.47; vs. PCV: $P = 0.0083$, OR = 0.53).

Conclusions. *C2/CFB* variants play a protective role in the risk of developing neovascular AMD and PCV in the Japanese. (*Invest Ophthalmol Vis Sci.* 2012;53:794-798) DOI:10.1167/iovs.11-8468

Age-related macular degeneration (AMD) is the leading cause of visual impairment in the elderly and the most common cause of blindness in developed countries.[1] Recent studies of the genetics of AMD have recognized it as a complex disease caused by the actions and interactions of several genes and environmental factors.[2-10] The three major AMD-associated loci in Caucasians include (1) the age-related maculopathy susceptibility 2 and high-temperature requirement factor A1 (*ARMS2/HTRA1*) gene[2,3] (2) the complement factor H (*CFH*) gene,[4-7] and (3) the complement component 2 and factor B (*C2/CFB*) gene loci.[8]

Some studies have reported that inflammatory processes may play a central role in AMD by contributing to the formation of drusen,[11,12] with *C2* and *CFB* being involved in initiation of the alternative complement cascade and activation of the classic component pathway, respectively. Numerous reports using Caucasian cohorts hold the consensus that genetic variants across *C2/CFB* are involved in protection against AMD.[8,13-15] However, all previous reports that evaluated populations in East Asia have shown an absence of association of *C2/CFB* variants in developing AMD. Thus, it was concluded that these variants are less likely to be associated with the development of AMD in Asians.[16,17]

Polypoidal choroidal vasculopathy (PCV) is clinically classified as a specific type of AMD and is usually diagnosed by indocyanine green angiography.[18] The incidence of PCV in Asian populations with neovascular AMD has been reported to be high, accounting for 54.7% of Japanese AMD patients and 24.5% of Chinese patients, compared with only 8% to 13% of Caucasians.[19-21] Previous studies revealed several genes that are susceptible to the development of PCV.[22-24] However, almost all reported genetic risk factors for developing PCV are identical with those for the development of AMD, which suggests that AMD and PCV share, at least in part, the same genetic background. In fact, PCV has many similarities with neovascular AMD, including demography, pathology, and manifestation.[19,21,25]

There have been studies in which the association between PCV and *C2/CFB* was evaluated in Asian populations, in a relatively smaller cohort size (165 participants by Lee et al.[26] and 313 participants by Kondo et al.[27]), but the results were negative, leading to the conclusion that pathobiological differences between PCV and neovascular AMD were present. However, these studies seemed to be underpowered, and recently, in a Caucasian cohort, Lima et al.[28] showed a positive associ-

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Table 1. Characteristics of the Study Population

	Cases		Control
	tAMD (n = 455)	PCV (n = 581)	Healthy Individuals (n = 865)
Age, y			
Mean ± SD	75.59 ± 8.60	72.59 ± 8.13	48.22 ± 16.18
Sex, n (%)			
Men	330 (72.5)	420 (72.3)	431 (49.8)
Women	125 (27.5)	161 (27.7)	434 (50.2)
Smoking, n (%)			
Never	151 (36.7)	200 (38.5)	454 (52.7)
Ever	261 (63.3)	319 (61.5)	408 (47.3)

ation in *C2/CFB* variants of the risk for PCV. Although PCV is more common in Asians than in Caucasians, the association between *C2/CFB* and PCV has not been subjected to detailed evaluation in Asian cohorts. The purpose of this study was to investigate whether the *C2/CFB* variants play a significant role in development of typical AMD or PCV by using almost 2000 participants from a Japanese population.

Methods

All procedures in this study adhered to the tenets of the Declaration of Helsinki, and the Ethics Committee of each institute involved approved the study protocols. All the patients were fully informed about the purpose and procedures of the study, with each patient providing written consent.

Four hundred and fifty-five patients with typical AMD (tAMD) and 581 patients with PCV were recruited from the Department of Ophthalmology at Kyoto University Hospital, Fukushima Medical University Hospital, and Kobe City Medical Center General Hospital. We used 865 healthy Japanese individuals, recruited from the Aichi Cancer Center Research Institute, as control subjects. They were recruited from first-visit outpatients after it was confirmed that they did not have cancer according to the cancer registry, medical record, and self-report. We recruited them without ophthalmic data and evaluated them as general population controls. When we found a significant association in a studied variant using the general population controls, we confirmed the association using a second control group comprising 336 elderly individuals who had received cataract surgery without age-related maculopathy (ARM), recruited from the Department of Ophthalmology at Kyoto University Hospital, Ozaki Eye Hospital, Japanese Red Cross Otsu Hospital, and Nagahama City Hospital. By funduscopic examination, the cataract control samples were confirmed not to have any drusen or pigment change. The definitions of exudative AMD and ARM were based on those of the International Classification System for ARM and AMD.[29] As proposed by the Japanese Study Group of Polypoidal Choroidal Vasculopathy,[30] the diagnosis of PCV was based on indocyanine green angiography, which showed a branching vascular network terminating in polypoidal swelling. tAMD showed classic CNV, occult CNV, or both. Patients with the following status were excluded from the study subjects: (1) high myopia (spherical equivalent, < -6.00 D), (2) geographic atrophy or drusen only, (3) an eye with both typical choroidal neovascularization and polypoidal lesions, and (4) an old lesion without a clear diagnosis. All diagnoses were made by three retina specialists (KY, AT, and AO); a fourth specialist (NY) was consulted when the subtype classification could not be decided on by the initial three reviewers. All subjects in the present study were unrelated and of Japanese descent.

Information on smoking status was obtained via a self-report questionnaire, with the three categories of never smoker, former smoker, and current smoker. The never smokers were those who had smoked fewer than 100 cigarettes in the past, current smokers were those who had smoked in the past 1 year, and former smokers were those who had quit smoking more than 1 year earlier. As in our previous study,[31] we combined the current smokers and the former smokers into ever smokers; thus, we analyzed the smoking status based on the two groups of never smokers and ever smokers.

We targeted *C2* rs547154 (IVS10) and *CFB* rs2072633 (IVS17), which have been described as having a positive association with the development of AMD in prior studies.[8,13,14,32] We analyzed two additional single-nucleotide polymorphisms (SNPs) on *CFB* (rs541862 and rs4151672) because they had relatively higher allele frequencies on the *C2/CFB* locus. Genomic DNAs were prepared from peripheral blood by using a DNA extraction kit (QuickGene-610L; Fujifilm, Minato, Tokyo, Japan). All case samples and cataract samples were then genotyped (Taqman SNP assay with the PRISM 7700 system; Applied Biosystems, Inc. [ABI], Foster City, CA). Individuals recruited from the Aichi Cancer Center Research Institute were genotyped with another system (Human-Hap610 chips; Illumina Inc., San Diego, CA).

Deviations in genotype distributions from the Hardy-Weinberg equilibrium (HWE) were assessed with the HWE exact test. Statistical analyses for differences in the observed genotypic distribution were performed by logistic regression analysis for age and sex adjustments. Haploview software was used to perform haplotype analysis and to infer the linkage disequilibrium (LD) among the evaluated SNPs.[33] P value correction was performed with the Bonferroni method, using the ratio of the number of selected SNPs across a gene. $P < 0.05$ was considered statistically significant.

Results

Demographics of the study population are shown in Table 1. Genotype and allele frequencies of the four SNPs were analyzed in the 455 patients with tAMD and 581 patients with PCV and compared with those of 865 healthy Japanese individuals. Details of allele frequencies and summary statistics are shown in Table 2. The genotyping of all evaluated SNPs was more than 98.2% successful, and the distributions of the genotypes for all study groups were in HWE ($P > 0.05$). In logistic regression analyses adjusted for age and sex, *C2* rs547154 and *CFB* rs541862 were significantly associated with both tAMD

Table 2. Distribution of Genotypes and the Results of the Association Tests

			Minor Allele Frequency			Association Test Results					
			tAMD	PCV	Control	Control vs. tAMD		Control vs. PCV		PCV vs. tAMD	
		Minor Allele	n = 455	n = 581	n = 865	P*	OR (95% CI)*	P*	OR (95% CI)*	P*	OR (95% CI)*
C2	rs547154	T	0.045	0.052	0.076	0.018	0.57 (0.35–0.91)	0.0062	0.54 (0.35–0.84)	0.385	0.83 (0.56–1.25)
CFB	rs541862	C	0.044	0.053	0.076	0.016	0.56 (0.35–0.90)	0.0061	0.54 (0.35–0.84)	0.317	0.81 (0.54–1.22)
CFB	rs2072633	A	0.436	0.413	0.454	0.875	0.98 (0.78–1.24)	0.240	0.88 (0.71–1.09)	0.425	1.08 (0.90–1.29)
CFB	rs4151672	T	0.018	0.022	0.024	0.845	0.92 (0.38–2.22)	0.842	0.93 (0.44–1.95)	0.584	0.84 (0.44–1.59)

* Adjusted for age and sex.

TABLE 3. Replication Study Using Cataract Patients

		Cataract Patients (n = 336)		vs. tAMD (n = 455)		vs. PCV (n = 579)	
		Minor Allele	MAF	P*	OR (95% CI)*	P*	OR (95% CI)*
C2	rs547154	T	0.092	0.0028	0.51 (0.33-0.79)	0.0038	0.56 (0.38-0.83)
CFB	rs541862	C	0.091	0.0028	0.51 (0.33-0.79)	0.0046	0.57 (0.38-0.84)

MAF, minor allele frequency in the cataract patients.
* Adjusted for age and sex.

(rs547154: P = 0.018, odds ratio [OR] = 0.57, 95% confidence interval [CI] = 0.35-0.91; rs541862: P = 0.016, OR = 0.56, 95% CI = 0.35-0.90) and PCV (rs547154: P = 0.0062, OR = 0.54, 95% CI = 0.35-0.84; rs541862: P = 0.0061, OR = 0.54, 95% CI = 0.35-0.84). These associations remained significant, even after a permutation procedure for multiple test correction (corrected $P < 0.05$). There was no SNP in the four tested SNPs across the *C2/CFB* locus, which showed a significant difference between tAMD and PCV.

Because the age of the controls was definitely younger than that of the cases, to adjust for a birth cohort effect, differential survival, or survivorship, we also performed a stratification analysis using 420 controls aged 50 years or older. This stratified cohort included 212 (50.5%) men, 208 (49.5%) women, 223 (53.3%) never smokers, and 195 (46.7%) ever smokers. The mean age ± SD of the group was 62.68 ± 7.67 years. We found that the associations of *C2* rs547154 and *CFB* rs541862 remained statistically significant in both tAMD (rs547154, P = 0.0048; rs541862, P = 0.0042) and PCV (rs547154, P = 0.0076; rs541862, P = 0.0075) in this stratification analysis, as well.

Next, we confirmed the positive associations using a second control cohort of 336 elderly cataract patients. The mean age ± SD of the cataract patients was 74.16 ± 8.42 years (range, 43- 94), and 142 (42.3%) male and 194 (57.7%) female patients were included. Table 3 shows the result of this replication analysis. Significant associations for developing both tAMD and PCV with *C2* rs547154 and *CFB* rs541862 were also shown in this evaluation ($P < 0.05$). In addition, we performed haplotype analysis using the cataract controls (Table 4). Haplotype analysis revealed that a common haplotype across the *C2/CFB* locus conferred a significant risk for both tAMD and PCV (P = 0.0030 and 0.0001, respectively) and a rare haplotype protectively associated with both tAMD and PCV (P = 0.0001 and 0.0016, respectively).

Finally, we conducted a logistic regression analysis that included the effects of the most robust Japanese AMD/PCV-associated variants, *ARMS2* A69S (rs10490924) and *CFH* I62V (rs800292), as well as age, sex, and smoking status. Because *C2* rs547154 and *CFB* rs541862 were in strong LD (pair-wise D′ = 1.0 and r^2 = 1.0), we analyzed rs547154 as the representative SNP of the *C2/CFB* locus. Table 5 shows the result of this logistic regression analysis. *C2/CFB* rs547154 remained significant both in tAMD and PCV, even after including the effects of these covariates (vs. tAMD: P = 0.0073, OR = 0.47, 95% CI = 0.27-0.82; vs. PCV: P = 0.0083, OR = 0.53, 95% CI = 0.33-0.85). After considering the effects of three major AMD-associated loci, we found that the effect of smoking was diminished in the risk for PCV (P = 0.292), and just a marginal association was found for tAMD (P = 0.0693).

DISCUSSION

The present study shows the significance of polymorphisms in *C2* and *CFB* for development of tAMD and PCV in a relatively large sample of Japanese patients. As a result of comparing the genotypic distributions of *C2/CFB* variants in a sample of Japanese patients with tAMD (n = 455) or PCV (n = 581) and in two independent control groups (865 healthy Japanese individuals and 336 cataract patients), we found that *C2* rs547154 (IVS10) and *CFB* rs541862 showed significant associations with the risk for both tAMD and PCV, with protective effects against the risk of the diseases.

Replication is the gold standard for assessing statistical results from genetic studies. However, a real result may fail to be replicated for numerous reasons, including inadequate sample size or variability in phenotype definitions across independent samples.[34] Although numerous reports have shown a significant association between *C2/CFB* variants and AMD in Caucasians, all studies in Asians have been unable to replicate these results.[16,17] However, in the present study, we clearly showed a significant association of *C2/CFB* variants for developing AMD in a Japanese cohort. Considering that minor allele frequency is similar between our cohort and previous reports, this discrepancy would be due to the small sample size used in the previous reports on Asian cohorts. Our study indicates that previous studies on the same subject did not reach statistical significance, and that large cohorts are needed to have enough statistical power to detect the association of the *C2/CFB* locus.

To date, all reports on Asian cohorts have shown a lack of association between *C2/CFB* polymorphism and PCV.[26,27] However, recently, Lima et al.[28] showed a positive association with PCV in Caucasians, even though incidence of PCV is lower in Caucasians than in Asians. In the present study,

TABLE 4. Distribution of Haplotypes and the Results of the Association Tests

Haplotype				Frequencies		P		
rs547154	rs541862	rs2072633	rs4151672	tAMD	PCV	Cataract Controls	Cataract vs. tAMD	Cataract vs. PCV
G	T	G	C	0.55	0.57	0.47	0.0030	0.0001
G	T	A	C	0.39	0.36	0.41	0.527	0.0467
T	C	A	C	0.044	0.053	0.092	0.0001	0.0016
G	T	G	T	0.018	0.022	0.029	0.109	0.264

tAMD, typical age-related macular degeneration.

TABLE 5. Comparison of the Significance of Major AMD-Associated Factors

	tAMD		PCV	
	*P**	OR (95% CI)	*P**	OR (95% CI)
Age, y	<0.0001	1.21 (1.18–1.24)	<0.0001	1.18 (1.15–1.20)
Sex, women/men	0.0127	1.87 (1.14–3.07)	<0.0001	2.64 (1.72–4.04)
ARMS2/HTRA1 rs10490924 (G/T)	<0.0001	2.43 (1.85–3.20)	<0.0001	2.02 (1.60–2.56)
CFH rs800292 (A/G)	<0.0001	1.89 (1.41–2.55)	<0.0001	2.02 (1.57–2.60)
C2/CFB rs547154 (G/T)	0.0073	0.47 (0.27–0.82)	0.0083	0.53 (0.33–0.85)
Smoking, never/ever	0.0693	1.56 (0.97–2.52)	0.292	1.25 (0.83–1.87)

* A logistic regression model was used to analyze the association between covariates.

C2/CFB variants were clearly associated with PCV. Our result is therefore in agreement with that of the Caucasian cohort used by Lima et al. Hence, our study also supports that there is no difference between tAMD and PCV in the role of *C2/CFB* for development of the disease.

In addition, we found the association of *C2/CFB* variants was unchanged, even when we adjusted for the effects of other established risk factors for AMD (age, sex, smoking, and a genetic background including *ARMS2* A69S and *CFH I62V*). In this study, common genetic variations at all three loci were associated with PCV, similar to that already documented in AMD—that is, SNPs that conferred a higher risk or protection from the disease in AMD were associated with the same in PCV. Furthermore, logistic regression analysis revealed that the role of environmental factors (smoking) diminishes when the effects of the three major AMD-associated loci (*ARMS2/HTRA1*, *CFH*, and *C2/CFB*) are taken into consideration. This result indicates that genetic factors have an enormous influence on whether people develop AMD and/or PCV. Among all covariates, *ARMS2/HTRA1* variants had the largest effect on the risk for tAMD (OR = 2.43), whereas sex had the largest effect on development of PCV (OR = 2.64). In a previous meta-analysis study,[35] the prevalence of late AMD in Asian women was reported to be much lower than in Asian men; on the other hand it is said that those with PCV are predominately male.[20] Considering the high prevalence of PCV in Asian populations, these results suggest that men would be more likely to develop PCV. In our study, genetic factors had important roles in the development of both tAMD and PCV. Thus, our results indicate that differences in sex would affect phenotypic differences in AMD.

In the present study, we evaluated different SNPs from those examined in the original study,[8] because minor allele frequencies of the SNPs evaluated in Caucasians were extremely low in the Japanese. To confirm the association reported in Caucasians, we also genotyped *C2* rs9332739, reported to be positive in the original study in cataract controls. However, there was no significant association in *C2* rs9332739 for development of tAMD and PCV in our cohort, because of its low allele frequency (data not shown), and *C2* rs9332739 did not have an impact on the result of haplotype analysis

We also grouped the current smokers and the former smokers into ever smokers, because this group had the highest tendency to develop PCV.[31] However, smoking was not found to have a significant independent association with the development of either tAMD or PCV in this study. Considering that smoking status was obviously different between the cases and the controls, this association should reach statistical significance if the number of participants were increased. Another reason for the lack of association could be because of the heterogeneity of smoking status. As with the general trend, the former smokers were older than others, and more men than women had smoked in our cohort (data not shown). In addition, we could not exclude the possibility that there were interactions between genetic and environmental factors or between genes; several studies have reported the presence of interactions between *ARMS2/HTRA1*, *CFH*, and smoking in AMD[9,10,36] and PCV.[31] Hence, further studies should be performed to ascertain the effects of interaction of different risk factors in the development of disease, including AMD-associated genes and smoking.

Another limitation of the present study is the difference between the case and control samples. The control samples were definitely younger than those in the case group, which means that some of these young controls may develop AMD or PCV in the future. To exclude a potential confounder of genetic background in age, we confirmed that our results were unchanged, even after a stratification analysis adjusting for the difference in age. In addition, to avoid a sampling error, we performed a replication study using another control group of a much closer age to the cases (cataract patients without ARM) and found a significant association between *C2/CFB* variants and development of AMD/PCV. However, because the prevalence of late AMD in the Japanese population is reported to be 0.5%,[37] the magnitude of the statistical bias of an association analysis should be negligible. In addition, considering that the case-control association analyses using such subjects are less apt to be statistically significant, our positive results should be acceptable.

Recently, subretinal drusenoid deposits, called reticular pseudodrusen, were differentiated from soft drusen with spectral domain optical coherence tomography (SD-OCT)[38] and were reported to be associated with late AMD.[39,40] We also evaluated whether *C2/CFB* variants are associated with developing reticular pseudodrusen in a small number of participants (n = 91) who had SD-OCT and autofluorescence imaging. However, we could not find a significant association between *C2/CFB* variants and the incidence of reticular pseudodrusen (data not shown). Further studies are needed to ascertain the association between *C2/CFB* variants and developing reticular pseudodrusen.

In conclusion, this study provides the first evidence that *C2/CFB* variants play a role in the risk of both neovascular AMD and PCV in Asians. Inflammation plays a central role in the pathobiology of AMD, with *C2* and *CFB* both encoding regulatory proteins that activate the complement pathway. As the inhibition of CFB with a specific chemical binding entity has been suggested to be a viable approach for the treatment of neovascular AMD,[41] our findings may suggest the potential effectiveness of such treatments by using anti-inflammatory agents, not only for AMD but also for PCV.

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Association of Elastin Gene Polymorphism to Age-Related Macular Degeneration and Polypoidal Choroidal Vasculopathy

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Purpose. To see if there is an association in Japanese between elastin gene (*ELN*) polymorphisms and neovascular age-related macular degeneration (AMD) or its subtypes, typical AMD (tAMD) and polypoidal choroidal vasculopathy (PCV).

Methods. The authors genotyped five single nucleotide polymorphisms (SNPs), rs2301995, rs2856728, rs868005, rs884843, and rs13239907, at Kyoto University and Saitama Medical University. A case-control study was performed on 1296 patients with AMD and 478 controls.

Results. A statistically significant association was detected between the rs2301995 SNP and AMD ($P = 0.018$). Furthermore, subtype analysis revealed a significant association of rs2301995 with tAMD ($P = 0.0018$), but not with PCV. The genotype distribution of rs2301995 also differed significantly between tAMD and PCV ($P = 0.00030$). The trend in genotype distribution of rs2301995 was similar between the Kyoto and the Saitama studies. The A allele frequency was higher in tAMD, whereas it was similar in PCV and in controls, which is opposite to that reported in a previous study that the A allele frequency is higher in PCV, whereas it is similar in tAMD and in controls. Haplotype analysis also showed that the *ELN* polymorphism is significantly associated with tAMD ($P = 0.0055$), but not with PCV.

Conclusions. *ELN* is associated with AMD in Japanese. Furthermore, the findings suggest that *ELN* is a susceptibility gene for tAMD but not for PCV, which is opposite to that reported in a previous study that *ELN* is the susceptibility gene for PCV but not for tAMD. (*Invest Ophthalmol Vis Sci.* 2011;52:8780-8784) DOI:10.1167/iovs.11-8205

Neovascular age-related macular degeneration (AMD) is divided into polypoidal choroidal vasculopathy (PCV), retinal angiomatous proliferation, and typical age-related macular degeneration (tAMD) that includes predominantly classic choroidal neovascularization (CNV) type, minimally classic CNV type, and occult with no classic CNV type. Differences between tAMD and PCV have been investigated for more than a decade. Although PCV is regarded as a subtype of AMD, its prognosis has been reported to be better than that of tAMD.[1,2] Furthermore, the response to treatment differs between tAMD and PCV. It has been shown that photodynamic therapy is more effective for PCV than that for tAMD.[3-5] In contrast, anti-vascular endothelium growth factor (VEGF) treatment just barely inactivates the polypoidal lesions of PCV, although it works well on the CNV associated with tAMD.[6-9] The prevalence of PCV and tAMD also differs depending on ethnicity. In Caucasians, PCV is seen in only 8-13% of neovascular AMD,[2] whereas it has been shown that 41.3-54.7% of neovascular AMD in Japanese patients have PCV.[10,11] These differences suggest genetic background differences in the development of tAMD and PCV.

Susceptibility genes for AMD have been investigated intensely, and evidence has shown that the *CFH* gene and the *ARMS2/HTRA1* gene are associated with AMD, tAMD, and PCV.[12-18] We have recently shown that the association of the *ARMS2* gene is stronger for tAMD than for PCV, whereas the association of the *CFH* gene is similar between tAMD and PCV.[19] Discovery of a susceptibility gene associated only with tAMD but not with PCV, or vice versa, would deepen our understanding of the difference in pathogenesis between tAMD and PCV.

Kondo and colleagues[20] evaluated five tag SNPs of the elastin gene (*ELN*) in Japanese, and showed that its polymorphism is associated with PCV but not with tAMD. Although they discussed the possibility that they might have underestimated the association with tAMD because of a type I error due to low samples sizes ($n = 285$), *ELN* might explain the different mechanisms involved in the development of tAMD and PCV. In Caucasians, however, it has been shown that *ELN* is not associated with PCV or AMD.[21,22]

Bruch's membrane is an elastin-rich extracellular matrix between retinal pigment epithelium and choroidal capillaries, whose defect can lead to CNV formation. Elastin polymerization deficit has been shown to lead to larger CNV in laser-

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TABLE 1. Characteristics of Study Population

Factor	Kyoto			Saitama		
	Controls	tAMD	PCV	Controls	tAMD	PCV
Number	336	408	518	142	216	154
Mean age ± SD, y	74.2 ± 8.4*	77.7 ± 8.4*	75.1 ± 8.5*	68.4 ± 9.8†	72.7 ± 8.7†	71.8 ± 7.8†
Sex, male/female	142/194‡	293/115‡	381/137‡	74/68‡	158/58‡	122/32‡

* $P < 0.0032$ each other with the Scheffé post hoc test.
† $P < 0.00031$ each other with the Scheffé post hoc test, except for tAMD and PCV ($P = 0.59$).
‡ $P < 0.0001$ with χ^2-test.

induced CNV in mouse.[23] Furthermore, histophathologic studies showed disruption of the elastic layer of polypoidal vessels in PCV. Evaluation of elastin in CNV development would lead to more precise understanding of the difference between tAMD and PCV.[24,25] In the present study we evaluated the five tag SNPs of *ELN* at Kyoto University and, separately, at Saitama University, using the same probes to examine the association of *ELN* to AMD and to its subtypes tAMD and PCV in a relatively large cohort ($n = 1774$).

MATERIALS AND METHODS

This study was performed in accordance with the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board/Ethics Committee of Kyoto Graduate School of Medicine, Kyoto, Japan, and by the Ethics Committee of Saitama Medical University, Saitama, Japan. Written informed consent was obtained from each patient.

In all, 926 patients with neovascular AMD and 336 patients with cataract but no AMD selected as control were recruited from the Department of Ophthalmology at Kyoto University Hospital and from the Ophthalmology Department at Fukushima Medical University Hospital. Of the 926 patients, 408 had tAMD and 518 had PCV. A total of 142 control subjects and 370 patients with neovascular AMD, including 216 with tAMD and 154 with PCV, were recruited from Saitama Medical University Hospital. All subjects in the present study were unrelated and all were of Japanese descent. The diagnosis of PCV was based on indocyanine green angiography, which showed a branching vascular network that terminated in polypoidal swelling. Typical AMD showed classic CNV, occult CNV, or both. All diagnoses in the Kyoto study and the Saitama study were made by three retina specialists at each facility using fundus photograph, fluorescein and indocyanine green angiography, and optical coherence tomography; a fourth specialist was called on when the subtype classification could not be decided on by the initial three reviewers. Less than 5% of the diagnosis was made with the help of the fourth specialist.

Genomic DNAs were prepared from peripheral blood using a DNA extraction kit (QuickGene-610L; Fujifilm, Minato, Tokyo, Japan, or Wizard Genomic DNA Purification Kit; Promega, Madison, WI). Tag SNPs were selected by use of a commercial database (HapMap database phase 2 release 22) by the tagger pairwise method with an R^2 cutoff of 0.8 and minor allele frequency (MAF) cutoff of 0.2, which included the five SNPs (rs2301995, rs2856728, rs868005, rs884843, and rs13239907) that were previously examined,[20,21] and were genotyped using an SNP assay (TaqMan, with the ABI PRISM 7700 system or 7000 system; Applied Biosystems, Foster City, CA). Mean ages were compared using ANOVA with post hoc comparisons tested by the Scheffé procedure. Allele frequency and sex ratio were compared with the χ^2 test and the genotype distribution was compared with the χ^2 test for trend or its exact counterpart. Age and sex differences were adjusted with logistic regression analysis software (R software, provided in the public domain by the R Foundation for Statistical Computing, Vienna, Austria, available at http://www.r-project.org/) by fitting the number of minor allele carried as an ordinal covariate, and age and sex as continuous and categorical covariates. Values of $P < 0.05$ were considered to be statistically significant.

RESULTS

Demographics of the study population are shown in Table 1. The mean ages and sex ratios were significantly different between controls and cases at each facility. Table 2 shows the genotype distributions of rs2301995, rs2856728, rs868005, rs884843, and rs13239907 in both the Kyoto study and the Saitama study. Since the genotype frequencies were not significantly different between the Kyoto study and the Saitama study, we pooled the data to evaluate the association of the five SNPs to AMD, tAMD, and PCV.

A statistically significant association was detected between rs2301995 and AMD ($P = 0.018$; Table 3). When the subtypes of AMD were evaluated, rs2301995 was significantly associated with tAMD ($P = 0.0018$), with the A allele as a risk, but was not associated with PCV. In both the Kyoto and the Saitama studies, the frequencies of the minor allele of rs2301995 and rs2856728 were higher in tAMD, whereas they were similar in controls and in PCV. The genotype distributions of rs2301995 and rs2856728 were also significantly different between tAMD and PCV ($P = 0.00030$ and 0.00012).

TABLE 2. Genotype Distribution of *ELN* Gene SNPs in Control Subjects and in Patients with AMD, tAMD, or PCV in Kyoto and Saitama Studies

Study/Factor	rs2301995 AA/AG/GG	rs2856728 CC/CT/TT	rs868005 CC/CT/TT	rs884843 GG/GA/AA	rs13239907 GG/GA/AA
Kyoto					
Controls, *n* (%)	13/99/217 (4/30/66)	25/125/182 (8/38/54)	22/107/203 (7/32/61)	58/167/108 (17/51/32)	133/161/38 (40/49/11)
AMD, *n* (%)	50/307/558 (5/34/61)	87/341/479 (10/38/52)	38/324/555 (4/35/61)	170/478/262 (19/52/29)	389/407/118 (43/44/13)
tAMD, *n* (%)	28/148/227 (7/37/56)	44/157/198 (11/39/50)	15/136/255 (4/33/63)	80/216/105 (20/54/26)	171/183/48 (43/45/12)
PCV, *n* (%)	22/159/331 (4/31/65)	43/184/281 (8/36/56)	23/188/300 (45/37/59)	90/262/157 (18/51/31)	218/224/70 (43/43/14)
Saitama					
Controls, *n* (%)	5/37/100 (4/26/70)	10/47/82 (7/34/59)	5/28/63 (5/30/65)	17/76/45 (12/55/33)	55/70/15 (39/50/11)
AMD, *n* (%)	16/105/234 (5/30/65)	26/118/196 (8/35/57)	11/29/51 (12/32/56)	68/141/125 (20/42/38)	151/160/43 (43/45/12)
tAMD, *n* (%)	12/58/134 (6/28/66)	20/65/108 (10/34/56)	9/13/33 (16/24/60)	41/74/71 (22/40/38)	87/90/26 (43/44/13)
PCV, *n* (%)	4/47/100 (3/31/66)	6/53/88 (4/36/60)	2/16/18 (6/44/50)	27/67/54 (18/46/36)	64/70/17 (42/47/11)

TABLE 3. Association of rs2301995 and rs2856728 to AMD, tAMD, and PCV versus Controls

	Allele		Genotype		
SNP/Factor	*P* Value	OR (95% CI)	*P* Value	Adjusted *P**	Minor Allele
rs2301995					
AMD	0.041	1.22 (1.01-1.48)	0.044	0.018	A
tAMD	0.0028	1.38 (1.12-1.71)	0.0036	0.0018	
PCV	0.47	1.12 (0.73-1.72)	0.48	0.33	
rs2856728					
AMD	0.77	1.05 (0.76-1.45)	0.78	0.28	C
tAMD	0.37	1.18 (0.83-1.68)	0.40	0.089	
PCV	0.57	0.89 (0.61-1.32)	0.57	0.89	
rs868005					
AMD	0.86	0.98 (0.81-1.19)	0.86	0.58	C
tAMD	0.72	1.15 (0.89-1.47)	0.72	0.67	
PCV	0.55	0.99 (0.79-1.25)	0.55	0.50	
rs884843					
AMD	0.23	0.91 (0.78-1.08)	0.22	0.42	G
tAMD	0.098	0.86 (0.73-1.03)	0.094	0.11	
PCV	0.59	0.95 (0.81-1.173)	0.59	0.88	
rs13239907					
AMD	0.72	1.03 (0.88-1.20)	0.72	0.60	G
tAMD	0.66	1.04 (0.87-1.24)	0.66	0.51	
PCV	0.84	1.01 (0.85-1.21)	0.84	0.98	

OR, odds ratio; CI, confidence interval.
* Adjusted for age and sex with regression analysis.

Similarly to Kondo et al.,[20] we performed a haplotype analysis (Haploview) using three SNPs: rs868005, rs884843, and rs2301995. The estimated frequencies are shown in Table 4. A significant difference was noted for the T-G-A in an analysis between controls and tAMD ($P = 0.0055$) and also in an analysis between tAMD and PCV ($P = 0.014$).

DISCUSSION

In the study reported herein, we showed that the *ELN* polymorphism is associated significantly with AMD, and subtype analysis revealed that *ELN* is a susceptibility gene for tAMD but not for PCV. Our findings are consistent with those of a previous report by Kondo et al.[20] in that *ELN* is a susceptibility gene for AMD. However, in that report they showed that the *ELN* polymorphism is associated with PCV, but not with tAMD. Furthermore, they reported that MAFs of rs2301995 and rs2856728 were higher in PCV, whereas they were similar in controls and in tAMD (Table 5). This is opposite to our findings—that the MAFs of rs2301995 and rs2856728 are higher in tAMD and are similar in controls and in PCV. The genotype distributions of rs2301995 and rs2856728 had a similar trend between the Kyoto and Saitama studies. Although further investigation is needed, the consistent trend in genotype distribution in the Kyoto study and in the Saitama study suggests that *ELN* is a susceptibility gene for tAMD, but not for PCV.

In Caucasians, Lima et al.[21] evaluated the *ELN* rs2301995 polymorphism in 66 patients with PCV and in 368 unaffected controls, and showed that it was not associated with PCV. Ennis et al.[22] also evaluated three *ELN* SNPs, rs868005, rs2071307, and rs11770302, in 479 AMD patients and in 479 controls, and showed that *ELN* is not associated with AMD in Caucasians. According to the report by Lima et al.,[21] the MAFs of the rs2301995 SNP in Caucasians were 6.3% in PCV, 5.4% in AMD, and 7.1% in controls, which are relatively lower than the MAFs in Japanese, which are reported to be 14-17%. Evaluating associations of SNPs whose MAFs are low tends to lead to false-negative results. For example, many East Asian studies reported that *CFH* Y402H SNP, the most significantly associated gene locus for Caucasian AMD, is not associated with AMD.[26-31] More recent studies, performed with a larger cohort or meta-analysis, however, revealed an association of the Y402H SNP to AMD, tAMD, and PCV in East Asians.[19,32] Further study with a larger cohort might be required to investigate the association of *ELN* rs2301995 SNP in Caucasians.

In the present study, haplotype analysis also supports the theory that *ELN* is associated with tAMD, not with PCV, in Japanese. The A-G-T haplotype was more prevalent in tAMD than that in controls and, furthermore, was significantly more prevalent in tAMD than that in PCV. However, Kondo and associates[20] reported that the A-G-T haplotype was more prevalent in PCV than that in controls or tAMD. The difference

TABLE 4. Haplotype Distribution of *ELN* Gene between Controls and tAMD, Controls and PCV, and tAMD and PCV

	Haplotype Frequency			*P* Value*		
Haplotype	Controls	tAMD	PCV	Controls vs. tAMD	Controls vs. PCV	tAMD vs. PCV
TAG	0.58	0.53	0.56	0.045	0.56	0.12
CGG	0.23	0.21	0.22	0.45	0.84	0.50
TGA	0.18	0.23	0.19	0.0055	0.54	0.014
TGG	0.015	0.017	0.018	0.70	0.62	0.92

* Corrected *P* value by permutation test (number of iterations = 1,000,000).

TABLE 5. Comparison of rs2301995 and rs2856728 Minor Allele Frequency

	rs2301995 A Allele Frequency				rs2856728 C Allele Frequency			
Factor	**Kyoto**	**Saitama**	**Pooled**	**Kondo et al.[20]**	**Kyoto**	**Saitama**	**Pooled**	**Kondo et al.[20]**
Controls	0.19	0.17	0.18	0.15	0.26	0.24	0.26	0.24
tAMD	0.25*	0.20	0.24*	0.14	0.31	0.27	0.30*	0.18
PCV	0.20	0.18	0.19	0.26†	0.27	0.22	0.26	0.32†

* $P < 0.001$ against controls and PCV.
† $P < 0.001$ against controls and tAMD.

between our findings and the previous report by Kondo et al.[20] might stem from selection bias or diagnosis difference among facilities. Furthermore, the association detected in our study and the previous study might be false-positive findings. In the present study, only one SNP of rs2301995 showed a significant association. When Bonferroni correction was applied, the *P* values of rs2301995 between control and tAMD become 0.042 in allele analysis and 0.027 in genotype analysis. The *P* value of the A-G-T haplotype between control and tAMD becomes 0.017. Further evaluation by other facilities would be indispensable.

The differences between tAMD and PCV in prognosis, reaction to treatments, and prevalence according to ethnicity suggest to us that the pathogenesis of tAMD and PCV is different, at least in part. Histopathologic studies have evaluated the pathologic features of PCV, and whereas some studies showed that the choroidal neovascularization of PCV is the same as that of tAMD, others showed that PCV consists of abnormalities of the inner choroidal vessels, and these differences have not led to a definitive understanding of the pathogenesis of these diseases.[24,25,33–38] Nakashizuka et al.[38] reported a lack of VEGF positivity in the vascular endothelial cells of PCV. In contrast, two other reports have shown the expression of VEGF in PCV[35,36] to be the same as that in CNV membranes secondary to tAMD.[39] Considered together with the relatively poor effects that anti-VEGF treatment have on PCV, *VEGF* gene polymorphism seems to be an attractive candidate in which to explore the difference between tAMD and PCV. However, evaluation of a previous report indicates no difference in *VEGF* gene polymorphism between tAMD and PCV.[11] Although the association difference of *ELN* to tAMD and PCV needs to be carefully judged from both the present study and previous studies, histopathologic studies of elastin expression in tAMD and PCV might reveal a difference in the pathogenesis between PCV and tAMD.

In summary, our findings indicate an association of the elastin gene variant with tAMD, but not with PCV, in Japanese subjects, but, since our findings are opposite those of previous studies, further investigation is warranted, and a genetic study may well deepen our understanding of the pathogenesis of tAMD and PCV.

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Antiviral Combination Therapy With Peginterferon and Ribavirin Does not Induce a Therapeutically Resistant Mutation in the HCV Core Region Regardless of Genetic Polymorphism Near the *IL28B* Gene

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An association has been reported between genetic polymorphism near *IL28B* gene and the prevalence of mutation of hepatitis C virus (HCV) core region residue 70, both of which have been associated with a lack of virologic response to antiviral combination therapy with peginterferon (PEG-IFN) and ribavirin. This study investigated whether PEG-IFN/ribavirin combination therapy induces amino acid (AA) mutation at residue 70 of HCV and whether genetic polymorphism near *IL28B* gene affects it. AA substitutions at residue 70 of the HCV core region were measured and compared before and after combination therapy in 65 non-responders and 88 relapsers to the combination therapy. In three patients in whom both wild-type AA (arginine) and mutant-type AA (glutamine or histidine) were detected at residue 70 before treatment, only mutant-type AA was identified after treatment. In two patients who had wild-type AA solely before treatment, both wild-type and mutant-type AAs were identified at residue 70 after treatment. In five patients, in whom the AA had changed at residue 70 between before and after treatment, four patients carried the TT genotype at a polymorphic locus (rs8099917) near the *IL28B* gene and one carried the TG/GG genotype. No difference was found in the prevalence of this change of AA at residue 70 between the TT and the TG/GG genotype. Antiviral combination therapy with PEG-IFN and ribavirin does not appear to induce mutation of HCV core region residue 70 regardless of genetic polymorphism near the *IL28B* gene in Japanese patients infected with HCV genotype 1b. ***J. Med. Virol. 83:1559–1564, 2011.*** © 2011 Wiley-Liss, Inc.

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KEY WORDS: chronic hepatitis C; peginterferon and ribavirin; amino acid substitution of HCV core region residue 70; genetic polymorphisms near the *IL28B* gene; mutation; non-sustained virologic responder

INTRODUCTION

Hepatitis C virus (HCV) causes chronic infection that can result in chronic hepatitis, cirrhosis of the liver, and hepatocellular carcinoma [Niederau et al., 1998; Kenny-Walsh, 1999]. The current standard therapy for patients with chronic HCV infection is the combination therapy with peginterferon (PEG-IFN) and ribavirin [Ghany et al., 2009]. Although the current treatment regimen has markedly increased the rate of patients with sustained virologic response, which indicates the eradication of HCV, only approximately 50% of patients infected with HCV genotype 1 achieve a sustained virologic response.

Many studies have investigated the potential baseline host- or virus-related factors that are associated

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