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# Common Variants in *CDKN2B-AS1* Associated with Optic-Nerve Vulnerability of Glaucoma Identified by Genome-Wide Association Studies in Japanese

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## Abstract

**Background:** To date, only a small portion of the genetic variation for primary open-angle glaucoma (POAG), the major type of glaucoma, has been elucidated.

**Methods and Principal Findings:** We examined our two data sets of the genome-wide association studies (GWAS) derived from a total of 2,219 Japanese subjects. First, we performed a GWAS by analyzing 653,519 autosomal common single-nucleotide polymorphisms (SNPs) in 833 POAG patients and 686 controls. As a result, five variants that passed the Bonferroni correction were identified in *CDKN2B-AS1* on chromosome 9p21.3, which was already reported to be a significant locus in the Caucasian population. Moreover, we combined the data set with our previous GWAS data set derived from 411 POAG patients and 289 controls by the Mantel-Haenszel test, and all of the combined variants showed stronger association with POAG ( $P < 5.8 \times 10^{-10}$ ). We then subdivided the case groups into two subtypes based on the value of intraocular pressure (IOP)—POAG with high IOP (high pressure glaucoma, HPG) and that with normal IOP (normal pressure glaucoma, NPG)—and performed the GWAS using the two data sets, as the prevalence of NPG in Japanese is much higher than in Caucasians. The results suggested that the variants from the same *CDKN2B-AS1* locus were likely to be significant for NPG patients.

**Conclusions and Significance:** In this study, we successfully identified POAG-associated variants in the *CDKN2B-AS1* locus using a Japanese population, i.e., variants originally reported as being associated with the Caucasian population. Although we cannot rule out that the significance could be due to the differences in sample size between HPG and NPG, the variants could be associated specifically with the vulnerability of the optic nerve to IOP, which is useful for investigating the etiology of glaucoma.

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## Introduction

Glaucoma is a neurodegenerative ocular disease and one of the leading causes of irreversible blindness worldwide [1]. It is characterized by the progressive loss of retinal ganglion cells and optic nerve axons, resulting in visual field defects [2]. One of the well-known major risk factors for glaucoma is elevated intraocular pressure (IOP) [2]. Thus, the measurement of IOP is routinely involved in the diagnosis of glaucoma. In fact, the IOP level has been applied to subdivide the most common form of glaucoma, primary open-angle glaucoma (POAG), into two subtypes [3]: POAG with high ( $\geq 22$  mmHg) IOP (POAG/HPG, high pressure glaucoma; hereafter referred to as "HPG") and with normal

(<22 mmHg) IOP (POAG/NPG, normal pressure glaucoma; hereafter referred to as "NPG"). Interestingly, ~92% of the Japanese POAG patients are categorized into the NPG subtype [4], whereas ~41% of Caucasian POAG patients are categorized as NPG [5], thus showing a unique epidemiological distribution of Japanese patients compared with other ethnic groups.

Aside from IOP measurements, the diagnosis of glaucoma is commonly made by observing optic nerve degeneration and visual field defects by means of fundus examinations and visual field tests, respectively. In the case of HPG, early drug treatment to lower IOP immediately following the onset of visual field damage has been shown to be quite effective in slowing the irreversible progression toward blindness [6,7]. In contrast, since NPG

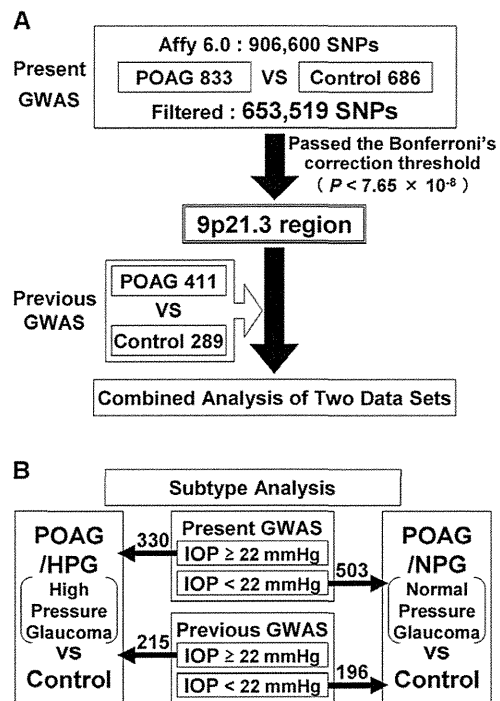
patients show normal IOP, they are often misdiagnosed. Therefore, both fundus examinations of the optic nerve and visual field tests are critical for the proper diagnosis of NPG patients. However, due to the restriction of the healthcare expenditure to include those examinations into a person's regular medical checkup, especially in the preclinical state of glaucoma, it would be of great benefit if the risk of developing glaucoma could be ascertained based on a simple blood test to assess the genetic markers for the disease.

Since glaucoma shows familial aggregation and its prevalence varies between individuals of different ethnicities, it has been theorized that genetic factors play a significant role in the pathogenesis of glaucoma [8,9]. Therefore, several institutions are making profound efforts to discover single-nucleotide variants for glaucoma by conducting a genome-wide association study (GWAS) [10–14], and there are a few published reports of the association of particular loci, such as the *CAV1/CAV2* locus (7q31.1) [13] and the *TMC01* or *CDKN2B-AS1* loci (1q24.1 or 9p21.3, respectively) [14], with POAG using Caucasian subjects. However, it appears that a controversy still exists as to determining the authentic variants associating with POAG, even within the same ethnicity of European descent [14,15]. We previously reported a GWAS and the subsequent follow-up study focused on the high-ranked variants identified in the initial population using in total of 1,575 Japanese subjects [10]. We identified six variants located in three loci, 1q43, 10p12.31, and 12q21.31, on the chromosome which were modestly associated with POAG, although the association results were not reproducible with a different population of different ethnicities [13,16,17]. In that previous study, we combined the patients from both HPG and NPG subtypes as a single case group in order to increase the statistical power [10]. Therefore, the genetic loci that were identified are most likely to be components of the molecular mechanism shared by both subtypes. Another Japanese group performed a GWAS [12] and discovered variants in *SRBD1* on 2p21 and *ELOVL5* on 6p12.1 that were associated with NPG (in that study, NPG was referred to as “normal tension glaucoma”) [18]. However, since the findings in that study were derived from a single population, replication studies to support those findings still need to be conducted.

In this present study, we examined two independent GWAS data sets, and then performed a meta-analysis by combining the data sets in order to discover authentic genetic markers for POAG, HPG, or NPG. We succeeded in identifying some significant variants in the *CDKN2B-AS1* locus using a Japanese population, as that locus has also been shown to be significantly associated with POAG in Caucasians [14]. Moreover, the variants that passed the Bonferroni correction seemed to be associated with POAG and POAG/NPG, thus suggesting that the locus potentially affects the NPG, rather than the HPG, subtype of POAG. Although we still need to confirm our findings by use of a larger HPG cohort in order to increase the statistical power, the variants identified in this study could be associated specifically with the vulnerability of the optic nerve to IOP, thus enabling us to investigate the etiology of glaucoma.

## Results

We first performed a GWAS in 833 POAG patients and 686 control subjects (hereafter referred to as “Present GWAS”; Figure 1A) who were selected and divided into case and control groups based on our strict diagnosis criteria. After genotyping, 653,519 SNPs passed the quality controls and were used for the association study (Figure 1A). In a quantile-quantile (Q-Q) plot

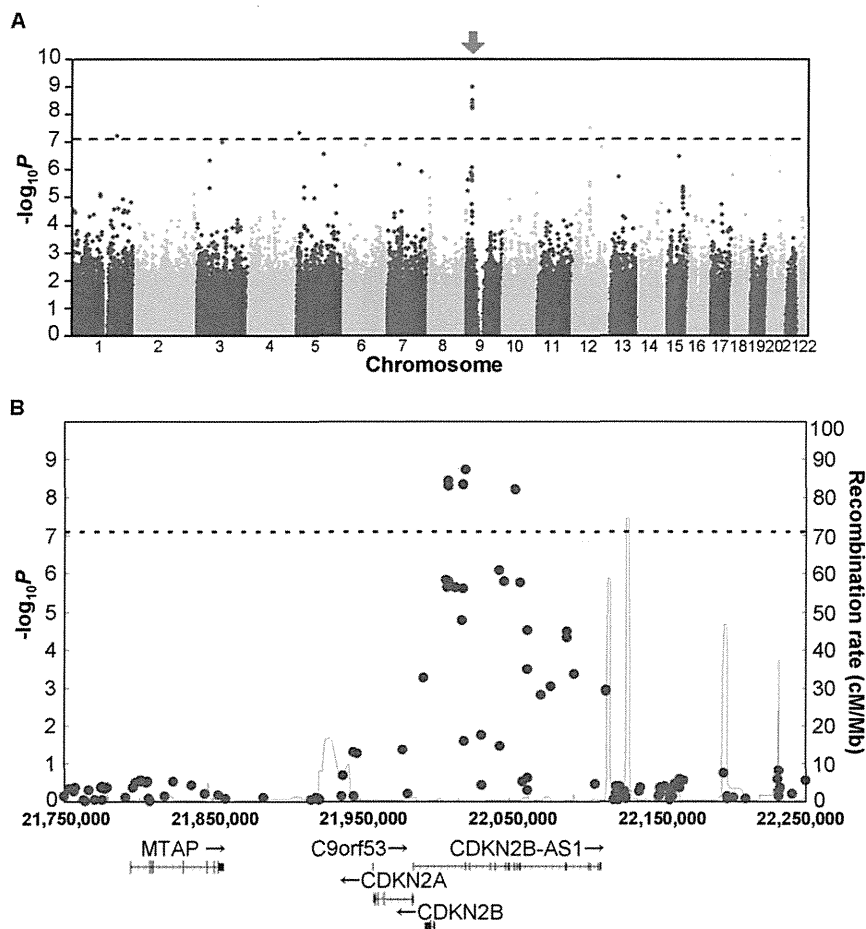


**Figure 1. Study design.** (A) We first performed the “Present GWAS” for POAG and identified the 9p21.3 locus. This result was confirmed by the combined analysis with the “Previous GWAS”. (B) We then subdivided the POAG subjects into two subtypes, POAG/HPG and POAG/NPG, and each group was analyzed by combining the two data sets in order to investigate the differences of statistical significance in the 9p21.3 locus.

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(Figure S1A), the genomic inflation factor ( $\lambda$ ) showed 1.021, suggesting that the population substructure should not have any substantial effects on the association analysis. Under these conditions, we obtained 8 genome-wide significant SNPs (Table S1) that passed the Bonferroni correction threshold ( $0.05/653,519 = 7.65 \times 10^{-8}$ ), of which 5 SNPs were located in *CDKN2B-AS1* on chromosome 9p21.3 (Figure 2). A few SNPs that showed modest to strong association were distributed across a single linkage disequilibrium (LD) block located in the locus (Figure S2). The remaining genome-wide significant SNPs identified from different chromosomes turned out to be genotyping errors due to the poor 2D clusters (Figure S3B–D).

Next, we updated the case and control groups of the previous GWAS population [10] with the latest clinical information and ended up with 411 POAG patients and 289 controls (hereafter referred to as “Previous GWAS”; Figure 1A). One SNP (rs8181047) of 5 genome-wide significant SNPs in *CDKN2B-AS1* identified from the Present GWAS was not able to be assessed in the Previous GWAS because a DNA probe of that particular SNP was not designed for the Affymetrix 500 K platform. Consequently, we combined the two sets of GWAS genotype data for the remaining 4 SNPs by use of the Mantel-Haenszel test [19], and the level of significance of all 4 SNPs increased from  $P < 5.2 \times 10^{-9}$  to  $P < 5.8 \times 10^{-10}$  (Table 1). These 4 SNPs were highly correlated with one another ( $r^2 > 0.98$ ), and were considered to have a similar effect for POAG based on the conditional analysis. These results suggested that it was effective to combine the data since the population stratification between the two data sets seemed to be ignorable, which was also supported by the results of the heterogeneity test. Moreover, we assessed the confounding effects



**Figure 2. Association results of the Present GWAS.** (A) The SNPs with a strong association appeared to exist in a cluster on chromosome 9 (red arrow). (B) The five significant SNPs that passed the Bonferroni correction threshold (horizontal dotted line) were identified in *CDKN2B-AS1* on the 9p21 locus. SNP positions followed the NCBI Build 36 coordinates. The genetic recombination rates (cM/Mb) estimated by HapMap Project [31] in Release 22 are indicated by the thin blue line.  
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for these SNPs with respect to age and gender, and none were found to be statistically significant (Table S2), thus suggesting that the obtained association results were specific to the case-control comparison. In addition, the two sets of GWAS data for 40 SNPs surrounding the significant 4 SNPs in the 9p21.3 locus were also combined and analyzed (Table S3, Table S4). Since these 44 SNPs, in total, were genotyped in both the Present and Previous GWAS, and all of the SNPs passed the genotyping quality

controls, these SNPs should prove useful in providing a broader overview of the significance of the locus. The results showed that the significance of combined SNPs was generally high between 22.0–22.1 Mb (Figure 3A). Within the particular locus, there seemed to be two distinct LD blocks; one in the side including genome-wide significant SNPs (“LD Block 1” in Figure 3D) and one in the other side with modestly associated SNPs (“LD Block 2” in Figure 3D).

**Table 1.** Association results of genome-wide significant SNPs in the Present GWAS.

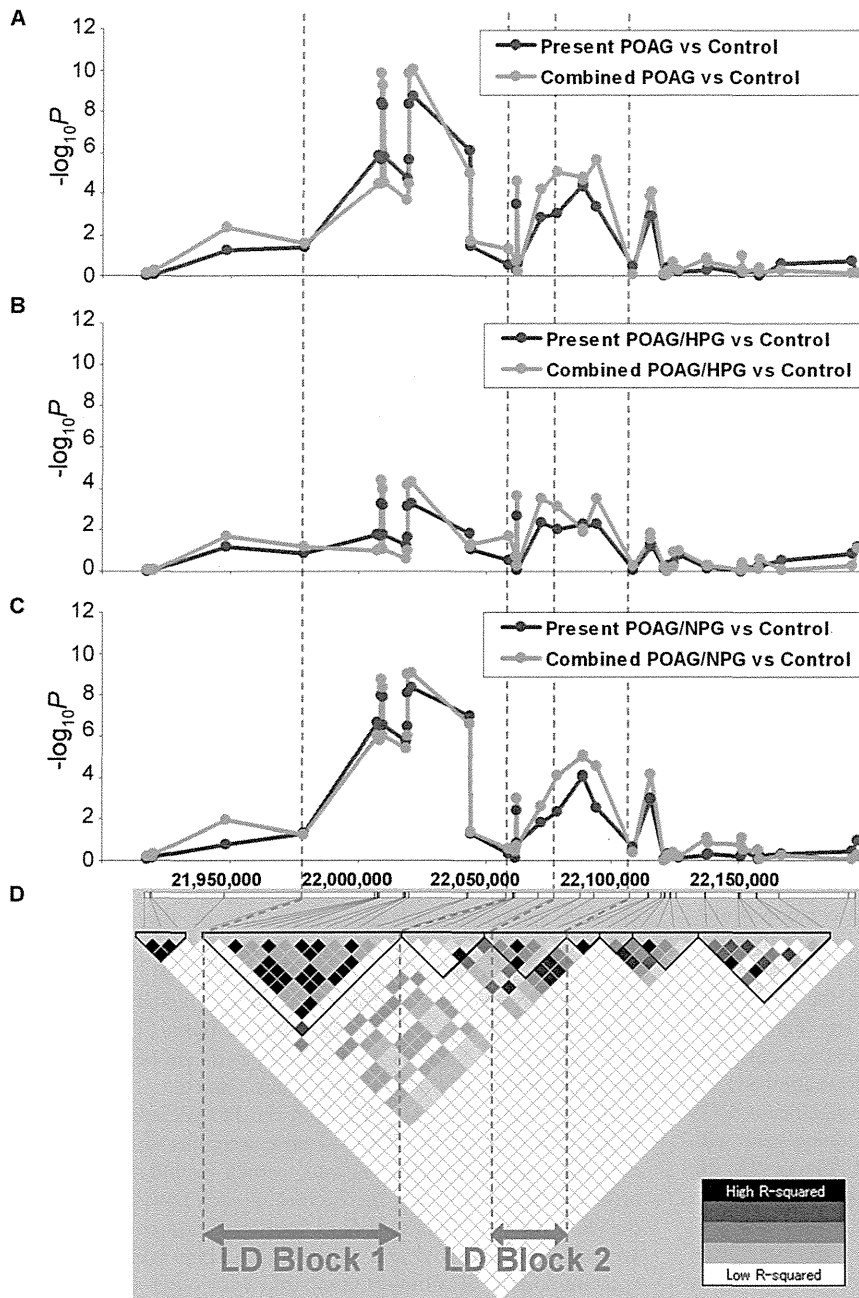
SNP:risk allele	Chr.	Position	Previous GWAS		Present GWAS		Previous & Present Combined				
			Frequency <sup>a</sup>	<i>P</i>	OR (95% CI)	Frequency <sup>a</sup>	<i>P</i>	<i>P</i> <sup>b</sup>	OR (95% CI)	Het- <i>P</i> <sup>c</sup>	
rs523096:A	9	22,009,129	0.89/0.84	$6.0 \times 10^{-3}$	1.56 (1.13–2.14)	0.89/0.82	$3.8 \times 10^{-9}$	1.86 (1.51–2.29)	$1.6 \times 10^{-10}$	1.76 (1.48–2.10)	0.42
rs518394:C	9	22,009,673	0.89/0.85	$1.5 \times 10^{-2}$	1.49 (1.08–2.07)	0.89/0.82	$5.2 \times 10^{-9}$	1.85 (1.50–2.28)	$5.8 \times 10^{-10}$	1.74 (1.46–2.07)	0.34
rs564398:A	9	22,019,547	0.90/0.85	$4.7 \times 10^{-3}$	1.58 (1.15–2.18)	0.90/0.82	$4.6 \times 10^{-9}$	1.86 (1.51–2.29)	$1.4 \times 10^{-10}$	1.77 (1.49–2.11)	0.47
rs7865618:A	9	22,021,005	0.90/0.85	$6.3 \times 10^{-3}$	1.56 (1.13–2.14)	0.90/0.82	$2.0 \times 10^{-9}$	1.88 (1.53–2.32)	$9.0 \times 10^{-11}$	1.78 (1.50–2.12)	0.39

<sup>a</sup>Risk allele frequency in POAG/controls.

<sup>b</sup>*P* value of combined 2 GWAS results by Mantel-Haenszel test.

<sup>c</sup>*P* value of Cochran’s Q heterogeneity test between Previous and Present GWAS.

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**Figure 3. Combined analysis of the Present and Previous GWAS data in 9p21.3 locus.** Three graphs representing the results of the association studies for POAG (A), HPG (B), and NPG (C) are shown in order from upper to lower, respectively. The results derived from the Present GWAS alone or the two combined GWAS data sets are indicated by the purple or green line graph plots, respectively. The lowest plot (D) shows an LD plot in the same region. All of the Present and Previous GWAS subject data was applied to draw the LD plot by Haploview v4.2. The color indicating pairwise  $r^2$  and LD block were lined by using the confidence interval (95%) of pairwise  $D'$ . Vertical dashed redline indicates the same region between the upper graphs and LD Block 1 and 2 at the lowest. doi:10.1371/journal.pone.0033389.g003

For the subtype analyses (Figure 1B), the POAG patients from both the Present and Previous GWAS populations were divided into two subtypes based on the clinical record of IOP measurement: 1) POAG patients with HPG (IOP  $\geq 22$  mmHg) and 2) those with NPG (i.e., patients who consistently showed an IOP of less than 22 mmHg) [3]. The number of subjects in each subtype was found to be 330 HPG and 503 NPG patients and 215 HPG and 196 NPG patients in the Present and Previous GWAS, respectively (Figure 1B). When we performed the analysis

separately using the Present GWAS data set, the Q-Q plots of HPG vs control and NPG vs control appeared to be quite different from one another (Figure S1B, C). In particular, the HPG results indicated non-deviated Q-Q plots (Figure S1B). In fact, although none of the SNPs were genome-wide significant for HPG (Figure S4A), we obtained 4 genome-wide significant SNPs that passed the Bonferroni correction threshold for NPG (Table S1, Figure S4B). As for NPG, the significance level of all 4 SNPs also increased when the two data sets were combined (Figure 3C, Table S3,

Table S4), however, the significance was still far from the Bonferroni threshold of significance in relation to HPG (Figure 3B, Table S3, Table S4). Although the significance level of SNPs residing in LD Block 2 showed slight differences among the different subtypes, the level in LD Block 1 seemed to be determining the difference of significance between the two subtypes, thus suggesting that the variants on LD Block 1 are closely associated with the susceptibility to glaucoma in the 9p21.3 locus (Figure 3D). Surprisingly, all of the significant SNPs from NPG overlapped with those obtained from POAG (Table S1). However, when we performed the heterogeneity test between the HPG and NPG groups, the results were not significant ( $P=0.21-0.25$ , Table S3), suggesting that the significant differences between the two subtypes could be due to the small sample size of the HPG group. Although we need to confirm the above results using a larger HPG cohort, these results suggested that the significant SNPs identified in *CDKN2B-AS1* on the 9p21.3 locus probably serve as genetic markers of glaucoma, which could be useful for investigating the etiology of glaucoma with respect to the vulnerability of the optic nerve to IOP.

## Discussion

In this study, we successfully identified genetic markers in *CDKN2B-AS1* strongly associated with POAG and POAG/NPG, but not with HPG, by analyzing two GWAS data sets using independent study populations totaling 2,219 Japanese subjects. For the case-control study of the Present GWAS, we excluded the samples without hesitation that didn't meet our strict quality control (see Supporting Information S1). Since we succeeded in excluding the samples possessing a fair amount of no-call or missed-call genotype data, our more stringent filters certainly improved the actual genotyping results for the association studies performed later. Thus, we were able to obtain an increased level of significance without any substructure effects of the two populations based on the definite diagnosis when combining the genotyping results of the Present and Previous GWAS data sets (Figure 3).

Using our polished data sets derived from distinct case and control subjects (see Methods and Supporting Information S1), we succeeded in identifying a cluster of genome-wide significant SNPs associated with POAG in *CDKN2B-AS1*, a non-coding gene with an unknown function, on chromosome 9p21.3 (Figure 2A, B). Several variants in chromosome 9p21 have been found to be associated with a variety of common diseases, and 9p21 was initially identified as a locus for coronary artery disease (CAD) [20–23] and type-2 diabetes [24–26]. However, little is known about the biological meanings underlying the locus, as 9p21 is a “gene desert” locus and most of the variants identified were non-coding. Recently, Harismendy et al. [27] reported that they have identified 33 enhancers in 9p21, some of which being within *CDKN2B-AS1*, and it turned out to be the second densest gene desert for predicted enhancers, thus suggesting the regulatory role of sequences residing within non-coding loci. They finally determined a few adjacent, as well as distant (>45 kb), target gene regions relevant to CAD biology physically interacting with the enhancer by 3D-DSL (also known as “4C”), a chromatin conformation capture technology, in human vascular endothelial cells. Overall, their study has provided an excellent example of a solution to link the unknown meanings of statistical association to a biological function. Since the POAG variants were also identified in *CDKN2B-AS1*, the variants would probably affect the expression level of the downstream genes *CDKN2A* and *CDKN2B*. In fact, Burdon et al. reported up-regulated *CDKN2A* and *CDKN2B* expression in response to the elevated IOP [14], suggesting the

involvement of the locus in molecular pathways leading to glaucoma development. Moreover, the possibility that the variants would also affect the distant unidentified target genes in the context of the complex etiology of glaucoma, as well as the nature of the identified locus [27], cannot be ruled out.

To date, several institutions have attempted to discover variants for glaucoma by conducting a GWAS, and a few published studies have reported the association of particular loci with POAG using Caucasian subjects (Table S5). Thorleifsson et al. reported the association of common variants near *CAVI/CAV2* on 7q31.1 using a population of European ancestry [13]. In contrast, Kuehn et al. reported that they failed to replicate their results in a United States cohort [15]. Moreover, meta-analysis of the association results derived from several institutions of Northern Europe, including the data from Thorleifsson et al. [13], identified a few new loci associated with POAG, including the *CDKN2B-AS1* locus on 9p21.3 [17]. Recently, the new loci at *TMC01* on 1q24.1 and *CDKN2B-AS1* were reported to be associated with Australian populations [14]. Thus far, only the association of the *CDKN2B-AS1* locus was replicated, even within the same ethnicity of European descent. Interestingly, Thorleifsson et al. also showed a modest association with the *CAVI/CAV2* locus using Chinese subjects [13], although the allele frequency of the particular variant in Asian subjects was quite low when compared with that in Caucasians, suggesting etiological differences due to the genetic background. In fact, according to the results of our Present GWAS for POAG, we were unable to replicate the association with the *CAVI/CAV2* and *TMC01* loci (Figure S5F, G, Table S5). Moreover, in the Present GWAS, we were unable to replicate the association results of the 6 SNPs identified in the previous study [10] (Table S5), even though the populations were of the same ethnic background, thus suggesting that we still need to discover authentic variants, irrespective of the difference in ethnicities, to elucidate the complex etiology of glaucoma. Consequently, it should be noted that the variants identified in the *CDKN2B-AS1* locus in this study using a Japanese population seemed to be shared with the Caucasian subjects (Table S5), thus showing that we have successfully obtained one of the authentic variants for POAG that is not ethnicity related.

In this study, we also subdivided the POAG subjects into two subtypes based on the IOP level measurements in an attempt to discover subtype-specific variants, which would be useful for investigating the different mechanism of each pathogenesis. Since the clinical states of both subtypes overlap almost completely, they are usually categorized as a single disease. However, as a unique epidemiological distribution compared with other ethnic groups, ~92% of the Japanese POAG patients fall into the NPG category, as determined by the Tajimi study [4], a robust epidemiology study. In fact, another Japanese group performed a GWAS focused on discovering NPG-specific variants [12]. They reported that the SNPs in *SRBD1* on 7q31.1 and *ELOVL5* on 6p12.1 were associated with NPG, although our group was unable to replicate those results (Figure S5D, E). On the contrary, we obtained an unexpected result that the variants associated with NPG were completely identical to those associated with POAG in *CDKN2B-AS1* identified in this study (Table S1). In contrast, none of the variants were significant for HPG (Figure 3B). Although we cannot rule out that the significance could be due to the differences in sample size between HPG and NPG cases according to the heterogeneity test ( $P=0.21-0.25$ ; Table S3), the results suggested that the genetic loci identified are most likely components of the molecular mechanism specific for NPG. It has been reported that there are differences in vulnerability of the optic nerve to IOP between HPG and NPG [18]. It has also been theorized that

*CDKN2B-AS1* affects the susceptibility of the optic nerve [14,28]. Since the variants identified in this study seemed to be shared among different ethnicities in functional aspects as well, the other variants should be contributing to the unique epidemiology of NPG in Japanese. By continuing to build upon the detailed investigation, such as obtaining in-depth sequencing data of the non-coding 9p21 locus, it might be possible to reveal not only the molecular mechanism of glaucoma pathogenesis but also the genetic diversity that resides within the locus among different ethnic backgrounds.

## Methods

### Ethics statement

This study was approved by the Institutional Review Board of Kyoto Prefectural University of Medicine and all procedures were conducted in accordance with the Declaration of Helsinki. All participants provided written informed consent after an explanation of the nature and possible consequences of the study.

### Case and control subjects for the Present GWAS

All participants were interviewed to determine their familial history of glaucoma and other ocular or general diseases. A total of 2,126 Japanese participants, including POAG patients, healthy volunteers, and patients with other ocular diseases, were recruited between March 2005 and December 2010 at the University Hospital of Kyoto Prefectural University of Medicine (Kyoto, Japan) to give peripheral blood samples for this study. A third person who was blind to both the blood sampling and genotyping assigned an anonymous code to each blood sample. Genomic DNA used for the genotyping experiments was isolated from the blood, and Epstein-Barr virus (EBV)-transformed lymphocytes were prepared from the remaining blood as previously described [29] to serve as the future resource of genomic DNA. POAG patients and controls suitable for this study were precisely selected based on the strict diagnosis as previously described [10]. In particular, subtype selection was performed by dividing POAG into two categories according to peak IOP without treatment; POAG/High Pressure Glaucoma (POAG/HPG, defined as  $\geq 22$  mmHg) and POAG/Normal Pressure Glaucoma (POAG/NPG) [3]. All of the diagnostic procedures, including case-control selection and subtype classification, were performed by three ophthalmologists (YI, MU, and KM) from a single institution. The age and female/male ratio of all of the subjects used in the Present and Previous GWAS are shown in Table S6. To examine the possible confounding effects of age and gender, we assessed the correlations between the values and the genotype data from the case and control samples by one-way ANOVA or chi-square test (Table S2).

### SNP genotyping and quality control for the Present GWAS

First, 906,600 SNPs were genotyped for 2,126 Japanese subjects by Genome-Wide Human SNP Array 6.0 (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions. As for the Present GWAS population, 839 POAG patients and 708 controls were selected after performing the quality control (QC) as described (Supporting Information S1). To exclude the potential inclusion of genetically related subjects into the population, identity-by-descent (IBD) estimates were performed for all possible combinations by PLINK v1.07 (<http://pngu.mgh.harvard.edu/~purcell/plink/>). In total, 6 POAG patients and 22 controls were assumed to be in first-degree relationships or in relationships with a few more-distant relatives, and were thus excluded from the

Present GWAS population. Finally, the Present GWAS population ended up with 833 POAG patients, comprised of 330 HPG and 503 NPG patients, and 686 controls. Population stratification for the present GWAS population was examined by principal component analysis using EIGENSTRAT software v3.0 (<http://genepath.med.harvard.edu/~reich/Software.htm>). As for the reference genomic population, the four HapMap populations (CEU, YRI, JPT, and CHB) were simultaneously applied to EIGENSTRAT. The generated cluster plots indicated that our POAG and control population was genetically clustered within the JPT population, and there was no outlier sample (Figure S6). We performed SNP quality control for the population on the autosomal SNPs based on the following QC filters: (i) call rate per SNPs in case and control samples  $\geq 95\%$ , (ii) minor allele frequency (MAF) in case and control samples  $\geq 1\%$ , and (iii) Hardy-Weinberg equilibrium (HWE) in control samples  $P \geq 0.001$ . Consequently, we analyzed the remaining 653,519 SNPs for the Present GWAS population.

### Population update for reanalyzing the Previous GWAS

Since 6 years had passed since the patients and healthy volunteers who participated in our Previous GWAS [10] were first recruited, we updated the case ( $n = 418$ ) and control ( $n = 300$ ) groups based on the latest clinical information. In total, 7 samples from the case group and 11 samples from the healthy control group were removed. The population for the reanalysis finally ended up with 411 POAG patients, comprised of 215 HPG and 196 NPG patients, and 289 controls. The Previous GWAS data was obtained by GeneChip® Human Mapping 500 K Array platform (Affymetrix) containing 500,568 SNPs. The quality control for the reanalysis was performed by using the same QC filters as used in the Present GWAS. Genotype data derived from the particular locus for combining with the Present GWAS data was extracted from these filtered SNPs.

### Data management and statistical analysis

To manage and analyze all of the genotype data, we used our in-house Genoika Server System (SASA Plus Co., Ltd., Fukuoka, Japan), which was well improved from the system used in the Previous GWAS [10,30] by the same system engineers. In addition, to manage the Previous GWAS data effectively, the Labo Server System (World Fusion Co., Ltd.) was used simultaneously. The Genoika Server System comes with PLINK v1.07 (<http://pngu.mgh.harvard.edu/~purcell/plink/>), the R program v 2.9.2 and 2.10.1 (<http://www.r-project.org/>), EIGENSTRAT software v3.0 (<http://genepath.med.harvard.edu/~reich/Software.htm>), and Haploview 4.2 (<http://www.broadinstitute.org/scientific-community/science/programs/medical-and-population-genetics/haploview/haploview>) built in, and all the analyses were performed by use of this system. In addition, Microsoft Office Excel 2003 (Microsoft Corporation, Redmond, WA) was used for preparing the data sets and statistical analysis. The frequency of alleles in the case and control samples was compared by use of the basic allele test. The odds ratio (OR) and the upper and lower limit of the 95% confidence interval (CI) of each SNP were calculated for the allele possessing a higher frequency in the case samples than in the control samples. The HWE was evaluated by the chi-square test. Q-Q plots were generated, by ranking the observed values from minimum to maximum and plotting them against their expected chi-square values using the "snpMatrix" package ver 1.14.6 in the R program (<http://www.r-project.org/>). We applied the Mantel-Haenszel test [19] to combine the data derived from the two data sets in order to reduce potential negative effects arising from the



biases in age and female/male ratio among the populations (Table S6).

## Supporting Information

**Figure S1 Q-Q plots for the Present GWAS.** Quantile-quantile (Q-Q) plots for the Present GWAS of POAG (A), HPG (B), and NPG (C) were generated by ranking the observed chi-square values from minimum to maximum and plotting them against their expected values. Genomic inflation factors ( $\lambda$ ) are also shown. These plots were created using the R-package snpMatrix. (PDF)

**Figure S2 LD plots in the 9p21 locus.** LD plots were generated from the Present GWAS data. The SNPs applied to these plots and the span of the region are the same as shown in Figure 2B. Upper and lower plots indicate the value of pairwise  $D'$  and  $r^2$ , respectively. The positions of 5 SNPs, which passed the Bonferroni correction threshold in the Present GWAS, are drawn in vertical dashed lines. These LD plots were generated using Haploview v4.2. (PDF)

**Figure S3 Genotyping error check.** 2D-cluster plots for the representative SNPs obtained from chromosome 9 (A), chromosome 1 (B), chromosome 5 (C), and chromosome 12 (D) that showed genome-wide significance. These 2D cluster plots were drawn by Genotyping Console 4.1 software (Affymetrix). (PDF)

**Figure S4 GWAS results of two subtypes.** Association results of the Present GWAS for the two subtypes of POAG, HPG (A) and NPG (B). The horizontal dashed line in each plot indicates the Bonferroni correction threshold for each study. (PDF)

**Figure S5 Evaluation of previously reported loci/genes.** Evaluation of the POAG-associated loci/genes reported previously using our Present GWAS data. The genes located in the loci are as follows: (A) ZP4, (B) PLXDC2, (C) TMTC2, (D) SRBD1, (E) ELOVL5, (F) CAV1/CAV2, and (G) TMC01. Each locus was evaluated by the results of POAG vs control, POAG/HPG vs control, and POAG/NPG vs control. The plotted SNPs were selected from QC filtered SNPs. Target genes are placed in the center of the 500-kb spanned region. Plots are shown with genomic annotation and HapMap LD (JPT+CHB) made by the UCSC Genome Browser (Human Mar. 2006). (PDF)

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**Figure S6 Population stratification analysis.** Population stratification analysis by EIGENSTRAT in the Present GWAS data set. POAG samples separated with HPG and NPG groups were applied. The version of HapMap reference data is release22 (Build36). Since Control, NPG, HPG, and HapMap JPT samples were tightly overlapped, only the plots for NPG (yellow cross) are visible. (PDF)

(PDF)

**Table S1 Genome-wide significant SNPs that passed the Bonferroni correction threshold in the Present GWAS.**

(PDF)

**Table S2 Analysis of confounding effects of age and sex for the candidate SNPs.**

(PDF)

**Table S3 Present GWAS results in 9p21.3 locus.**

(PDF)

**Table S4 Combined GWAS results in 9p21.3 locus.**

(PDF)

**Table S5 Association results of Present GWAS for previously reported SNPs.**

(PDF)

**Table S6 Characteristics of the samples.**

(PDF)

**Supporting Information S1 Quality control for genotyping.**

(PDF)

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Conceived and designed the experiments: SK KT. Performed the experiments: MN YI NO MU KI HA KM. Analyzed the data: MN YT SK KT. Contributed reagents/materials/analysis tools: YI MF MU KI MK KM SK. Wrote the paper: MN YI YT KM SK KT.



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in patients with psoriasis, in order to carry out a subgroup analysis.

The present findings suggest that high serum CRH level, possibly in response to stress, stimulates skin mast cells to release VEGF and contribute to skin inflammation evident in patients with severe psoriasis. Continuous or repeated stimulation may lead to decreased expression of skin CRHR-1, as we showed recently with cultured mast cells,<sup>9</sup> and hence decreased VEGF, as was evident in patients with mild disease. CRH and CRHR-1 may therefore participate in the pathogenesis of psoriasis and AD, especially when worsened with stress, through mast-cell activation to release VEGF. Mast-cell blockers may provide novel treatment approaches.

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## Epistatic interaction between Toll-like receptor 3 (TLR3) and prostaglandin E receptor 3 (PTGER3) genes

To the Editor:

We previously reported that conjunctival eosinophilic infiltration in murine experimental allergic conjunctivitis (EAC) was significantly less marked in Toll-like receptor 3 gene (*TLR3*) knockout (KO) mice<sup>1</sup> and significantly more marked in prostaglandin E receptor 3 (EP3) gene (*PTGER3*) KO mice than in wild-type mice.<sup>2</sup> Considering the opposite roles of *TLR3* and *EP3* in allergic conjunctivitis, we speculate the possibility of unknown functional interaction between *TLR3* and *EP3*.

Intriguingly, we have also reported that Stevens-Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN) accompanied by severe ocular surface complications was associated with *TLR3* gene polymorphisms<sup>3</sup> and *PTGER3* polymorphisms.<sup>4</sup> SJS is an acute inflammatory vesiculobullous reaction of the skin and mucosa, often including the ocular surface,<sup>5</sup> and TEN occurs with its progression. SJS/TEN with ocular surface complications often results in severe and definitive sequelae, such as vision loss (see Fig E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).<sup>6</sup>

For the past decade, single nucleotide polymorphisms (SNPs) have been widely used as genetic markers for identifying human disease-susceptibility genes. However, it has become apparent that gene-gene interactions should be considered in addition to major single-locus effects.<sup>7</sup> In particular, nonadditive (epistatic) models for some complex diseases fit to actual observations, suggesting interactions involving multiple loci.

In this study we examined whether there are functional interaction between *TLR3* and *EP3*. Moreover, we also examined whether there is an epistatic interaction between *TLR3* and *PTGER3* polymorphisms in patients with SJS/TEN with ocular surface complications.

This study was approved by the institutional review board of Kyoto Prefectural University of Medicine and the University of Tokyo, Graduate School of Medicine. All experimental procedures were conducted in accordance with the principles of the Helsinki Declaration. Details of the patients and methods are described in the Methods section in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org). The primers and probes used in this study are shown in Table E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org).

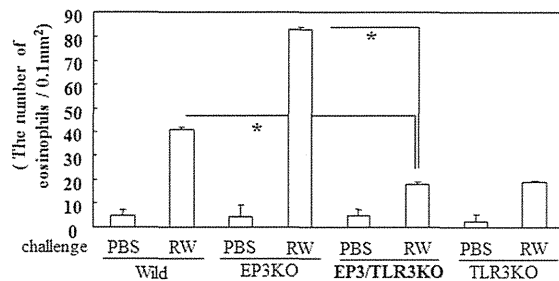
First, we examined the functional interaction between *TLR3* and *EP3* by using *TLR3* KO, *PTGER3* KO, and *TLR3/PTGER3* double-knockout (DKO) mice in addition to our EAC model.

We compared conjunctival eosinophil infiltration in wild-type, *TLR3* KO, *PTGER3* KO, and *TLR3/PTGER3* DKO mice. Although sensitization (intracutaneous and intraperitoneal injection of short ragweed pollen [RW; Polysciences, Inc, Warrington, Pa] adsorbed on aluminum hydroxide [200  $\mu$ g of RW and 2.6 mg of alum]) without challenge (RW eye drop) did not affect the number of eosinophils after sensitization and challenge, the number of eosinophils in the lamina propria mucosae of the conjunctiva was significantly increased in all of them compared with those in PBS-challenged control animals, and the number after sensitization and challenge in *PTGER3* KO mice was significantly larger and significantly lower in *TLR3* KO than in wild-type mice, as we have reported previously.<sup>1,2</sup> Because TLR3 could regulate allergic inflammation in the absence of exogenous viral infection or the TLR3 ligand, it is possible that in our allergic conjunctivitis model endogenous RNA from tissues or cells stimulates TLR3.<sup>1</sup> With respect to EP3, one of the prostaglandin E receptors (EP1-EP4), our earlier observations suggested that during the elicitation phase of our EAC model, prostaglandin E<sub>2</sub> is synthesized in the conjunctival epithelium through microsomal prostaglandin E synthase 1.<sup>2</sup>

Furthermore, in *TLR3/PTGER3* DKO mice the number of eosinophils in the lamina propria mucosae of the conjunctiva was decreased to a level similar to the number of eosinophils in the lamina propria mucosae of the conjunctiva in *TLR3* KO mice and was significantly lower than the number of eosinophils in the lamina propria mucosae of the conjunctiva in not only *PTGER3* KO mice but also in wild-type mice (Fig 1). In addition, we previously reported that in human conjunctival epithelial cells the EP3 agonist suppressed the production of cytokines, such as thymic stromal lymphopoietin<sup>8</sup> and RANTES,<sup>9</sup> induced by polyinosinic:polycytidylic acid, a TLR3 ligand. Thymic stromal lymphopoietin and RANTES play important roles in the recruitment of eosinophils. These results suggest that EP3 negatively regulates the eosinophilic infiltration of EAC induced by TLR3, which causes reduced eosinophilic conjunctival inflammation in *TLR3/PTGER3* DKO mice, despite the pronounced eosinophilic conjunctival inflammation seen in *PTGER3* KO mice.

We have reported that the frequency of carriers of the HLA-A\*0206 allele is significantly higher among Japanese patients with severe ocular surface complications.<sup>10</sup> We have also performed SNP association analysis of candidate genes and documented the associated polymorphisms of several immune-related genes, including *TLR3*,<sup>3</sup> IL-4 receptor (*IL4R*),<sup>11</sup> *IL13*, and Fas ligand (*FasL*) in Japanese patients with SJS/TEN. Furthermore, we have performed a genome-wide association study of the patients with SJS/TEN and found associations between 6 SNPs in the *PTGER3* gene and the Japanese patients with SJS/TEN.<sup>4</sup>

We carried out a statistical search for interactions between all possible pairs of loci by applying high-dimensional variable selection methods, such as Sure Independence Screening (SIS) and LASSO, to the comprehensive dataset obtained from our previous studies for a total of 14 immune-related genes (see Table E2 in this article's Online Repository at www.jacionline.org), including *PTGER3* and *TLR3*. After filtering with the standard SNP quality control filter, 36 SNPs were used for SIS to scan a total of  $5778 (3 \times 36 + 9 \times 36 \times [36-1]/2)$  dummy variables. As a result, iterative SIS reported 2 variables with susceptible effects on SJS, which were involved in locus pairs of *PTGER3-TLR3* and *HLA-A-IL1A*, respectively (see Table E3 in this article's Online Repository at www.jacionline.org). The result



**FIG 1.** Functional interaction between EP3 and TLR3. In *TLR3/PTGER3* DKO mice the number of eosinophils in the lamina propria mucosae of the conjunctiva was decreased to a level similar to that seen in *TLR3* KO mice and was significantly lower than that seen in either *PTGER3* KO or wild-type mice. Data are shown as means  $\pm$  SEMs of samples from all the mice examined (wild-type: phosphate-buffered saline, n = 24; RW, n = 28; *PTGER3* KO mice: phosphate-buffered saline, n = 23; RW, n = 25; EP3/TLR3 DKO mice: phosphate-buffered saline, n = 4; RW, n = 11; *TLR3* KO mice: phosphate-buffered saline, n = 12; RW, n = 12). \* $P < .0005$ .

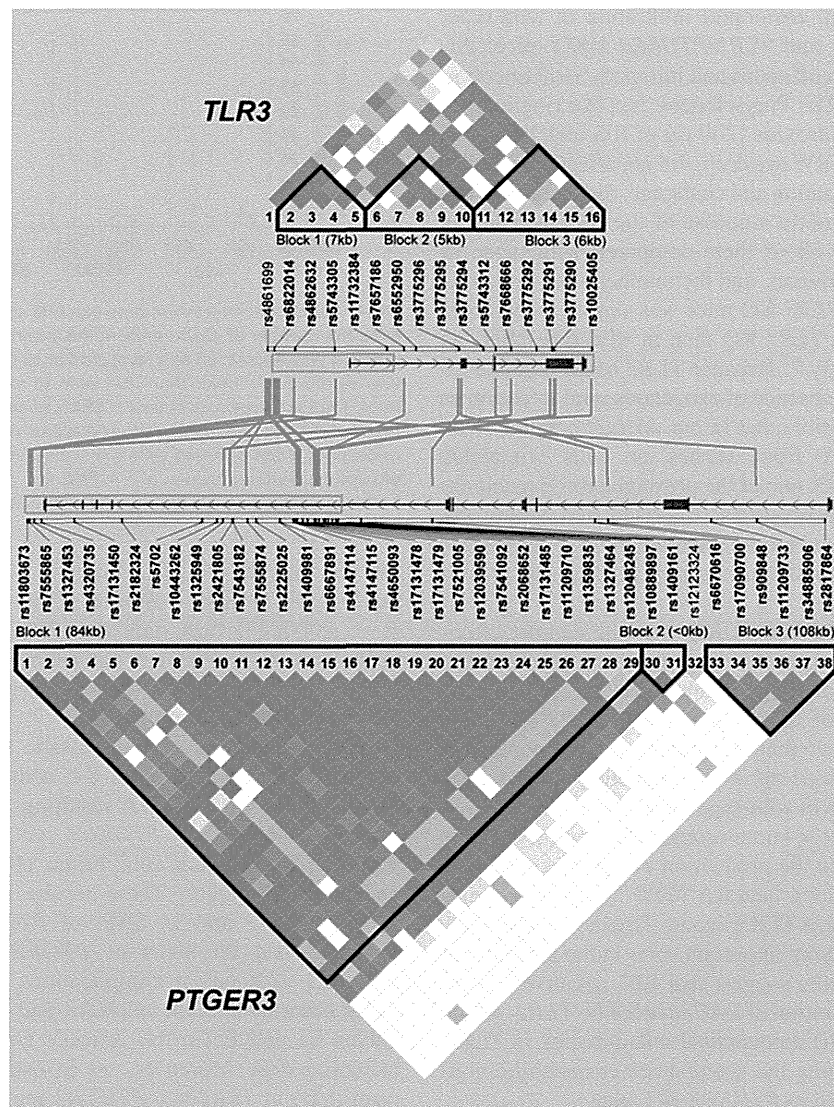
showed that the *PTGER3* rs.4147114G/C SNP with the *TLR3* rs.3775296T/T SNP exhibited a higher odds ratio (OR, 25.3;  $P = .0000527$ ) than only the *PTGER3* rs.4147114G/C SNP (OR, 2.66;  $P = .0023$ ) or only the *TLR3* rs.3775296T/T SNP (OR, 5.35;  $P = .00025$ ). These 2 susceptible interactions were also confirmed by using LASSO.

Next, we focused on the epistatic interaction between *TLR3* and *PTGER3* and analyzed the additional 10 SNPs of *TLR3* and 32 SNPs of *PTGER3*, resulting in a total of 17 SNPs of *TLR3* and 38 SNPs of *PTGER3*. All genotyping results agreed with Hardy-Weinberg equilibrium ( $P > .01$ ) in both the case and control samples. These results showed that 5 additional SNPs of *TLR3* and 14 SNPs of *PTGER3*, a total of 7 SNPs of *TLR3* and 20 SNPs of *PTGER3*, were associated with SJS/TEN with ocular complications in addition to the previously reported 2 SNPs of *TLR3* and 6 SNPs of *PTGER3* (see Tables E4 and E5 in this article's Online Repository at www.jacionline.org). Moreover, we investigated linkage disequilibrium (LD) in *TLR3* and *PTGER3* regions by using the squared correlation coefficient (1 SNP of *TLR3* [rs3775293] for which the minor allele frequency in both cases and control subjects was less than 5% was excluded) and identified 3 solid-spine LD blocks in each region. Iterative SIS reported 14 variables with nonzero regression coefficients as if connecting the 5' region of *TLR3* (block 1) and the 3' region of *PTGER3* (block 1, Fig 2).

We previously reported that the expression of EP3, the protein of the *PTGER3* gene, was downregulated in the conjunctival epithelium of patients with SJS/TEN with ocular surface complications.<sup>4,12</sup>

Although *TLR3* mRNA expression might also be downregulated in patients with SJS/TEN (see Fig E2 in this article's Online Repository at www.jacionline.org), our immunohistologic analysis did not clearly detect downregulation of the protein (see Fig E3 in this article's Online Repository at www.jacionline.org).

In the conjunctival epithelium of patients with SJS/TEN, EP3 was remarkably downregulated, and TLR3 might also be downregulated. Because EP3 might be more strongly downregulated than TLR3 in these patients, it is possible that EP3 is incapable of preventing TLR3-associated inflammation in patients with SJS/TEN.



**FIG 2.** LD in EP3 and TLR3 regions. LD in the *TLR3* and *PTGER3* regions show 3 solid-spine LD blocks in each region. Iterative SIS reported 14 variables with nonzero regression coefficients, as if connecting the 5' region of *TLR3* and the 3' region of *PTGER3*.

In conclusion, we have suggested the functional interaction between *TLR3* and EP3 supported by their epistatic interaction that confers an increased risk for SJS with severe ocular surface complications.

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## Esophageal eosinophilia caused by milk proteins: From suspicion to evidence based on 2 case reports

### To the Editor:

The First International Gastrointestinal Eosinophilic Research Symposium defined eosinophilic esophagitis (EoE) as a primary clinicopathologic disorder of the esophagus characterized by esophageal symptoms, upper gastrointestinal tract symptoms, or both in association with esophageal mucosal biopsy specimens containing more than 15 intraepithelial eosinophils/high-power field (hpf) in 1 or more biopsy specimens and absence of pathologic gastroesophageal reflux disease, as evidenced by a normal pH-monitoring study of the distal esophagus or lack of response to high-dose proton pump inhibitor medication.<sup>1,2</sup> EoE mostly occurs in male subjects (80%) and appears to have a common familial form and a strong association with atopy (70%). IgE and non-IgE immune mechanisms might cooperate in the pathogenesis of EoE, as supported by a series of recent studies.<sup>3</sup>

Evidence that food allergy causes EoE has been shown in children after an elemental diet (90% to 95% total resolution). A 6-food elimination diet produced a 74% overall improvement. Also, a diet based on skin test results showed a 69% reduction in symptoms.<sup>2,4</sup> Comparing these findings with those of our series of children (n = 17), avoidance of culprit foods identified by means of skin tests or specific IgE measurements achieved complete remission in 9 of 17 patients. Moreover, 3 patients achieved remission of symptoms with an elemental diet. The offending food was identified in 8 of 17 patients.<sup>5</sup>

Nevertheless, the role of food allergy in adults is less convincing than in children. Gonsalves et al attempted to treat 18 adult patients with EoE by adopting an elimination diet, as had previously succeeded in children.<sup>6</sup> Symptomatic improvement occurred in 94% of the adult patients, and the histology ameliorated in 50%. Cases of EoE in adults have also been resolved by eliminating dairy products.<sup>4</sup> Other authors found no correlation between allergy tests and the outcome of the elimination diet. In our series of adults with EoE, most had negative food-based skin test results and negative serum specific IgE levels and underwent swallowed inhaled corticosteroid treatment, with varying results.<sup>7</sup>

We describe the cases of 2 adult women with immediate symptoms limited to the esophagus after consuming milk products. In both cases morphologic changes compatible with acute esophageal eosinophilia were confirmed by means of endoscopy and biopsy.

Case 1, a 31-year-old woman previously given a diagnosis in our allergy division of rhinoconjunctivitis caused by aeroallergen hypersensitivity, oral allergy syndrome caused by fruit, and additional milk allergy, attended our outpatient clinic for a routine follow-up visit.

When the patient was 10 years of age, she started to have dysphagia 5 to 10 minutes after dairy product ingestion. Skin prick tests (SPTs) were performed against commercial standardized extracts of cow's milk, goat's milk, sheep's milk,  $\alpha$ -lactalbumin,  $\beta$ -lactoglobulin, and casein (ALK-Abelló, Madrid, Spain) by measuring the largest diameter of the wheal. The test result was considered positive if the wheal was 3 mm larger than that elicited by the negative control.

The patient had negative results against cow's milk,  $\alpha$ -lactalbumin, and  $\beta$ -lactoglobulin and positive results to casein (4 mm). Total IgE was 290 IU/mL. Tryptase was 2.43 IU/mL. Specific IgE (CAP; Phadia, Uppsala, Sweden) was 0.10 kU/L to cow's milk, 0.0 kU/L to  $\alpha$ -lactalbumin, 0.0 kU/L to  $\beta$ -lactoglobulin, 0.12 kU/L to casein, 0.09 kU/L to goat's milk, and 0.0 kU/L to sheep's milk. A complete battery of food patch testing was performed, including to cow's milk; however, the patient had negative results for all the foods tested.

Esophagogastroendoscopy performed that day revealed normal esophageal mucosa, and biopsy specimens of the proximal and distal esophagus were taken (3 samples from each location). Grouped blocks measured 0.3 × 0.2 × 0.2 cm and were stained with hematoxylin and eosin, showing 2 to 3 eosinophils per hpf. Immunohistochemistry was performed with anti-tryptase (CMA890; Cell Marque, Rocklin, Calif), eliciting a mean of 1 to 4 mastocytes per hpf (Fig 1, A and C).

We decided to perform a double-blind, placebo-controlled food challenge to cow's milk to assess milk tolerance. The patient reported pharyngeal pruritus and chest tightness, as well as dysphagia 15 to 20 minutes after milk ingestion. Given a suspected EoE diagnosis, informed consent was obtained for an esophagogastroendoscopy within 48 hours after milk intake, which revealed normal mucosa. Four biopsy specimens of the upper and lower parts of the esophagus were taken from the same location as before challenge. Grouped blocks measured 0.4 × 0.3 × 0.3 cm, and eosinophil and mastocyte counts were carried out manually by 2 experienced pathologists (one of them blinded). A minimum of 20 hpf were examined, subsequently calculating the mean of the total number of eosinophils

## METHODS

### Patients

This study was approved by the institutional review board of Kyoto Prefectural University of Medicine and the University of Tokyo, Graduate School of Medicine. All experimental procedures were conducted in accordance with the principles of the Helsinki Declaration. The purpose of the research and the experimental protocols were explained to all participants, and their prior written informed consent was obtained.

Diagnosis of SJS/TEN was based on a confirmed history of acute onset of high fever, serious mucocutaneous illness with skin eruptions, and involvement of at least 2 mucosal sites, including the ocular surface.

In the acute stage patients with SJS/TEN manifest vesiculobullous lesions of the skin (Fig E1, A) and mucosa (especially that of the eyes and mouth), severe conjunctivitis (Fig E1, B), and persistent corneal epithelial defects caused by ocular surface inflammation. Oral involvement, including blisters, erosions, and bleeding of the mouth and lips (Fig E1, C), has been observed in all patients with SJS/TEN with severe ocular surface complications, and almost all such patients lose their fingernails in the acute or subacute stage as a result of paronychia (Fig E1, D). In the chronic stage, despite healing of the skin lesions, ocular surface complications, including conjunctival invasion into the cornea (Fig E1, E), dry eyes, symblepharon, and in some instances keratinization of the ocular surface persist.

To investigate 44 SNPs of the 13 genes along with alleles of HLA-A analyzed by means of direct sequencing, we enrolled 61 patients with SJS/TEN in the chronic or subacute phase; all presented with symptoms of ocular surface complications. The control subjects were 130 healthy volunteers. All participants and volunteers were Japanese residing in Japan. The average age of the 61 patients and 160 control subjects was  $45.3 \pm 18.1$  (SD) and  $36.8 \pm 11.9$  (SD) years, respectively. The male/female ratios in the patient and control groups were 26/35 and 49/81, respectively.

Furthermore, we added 55 subjects and 91 control subjects to obtain a total of 116 case samples and 221 control samples for analysis of LD block around *TLR3* and *PTGER3*. The average age of the 116 patients and 221 control subjects was  $44.0 \pm 18.0$  (SD) and  $35.6 \pm 11.1$  (SD) years, respectively. The male/female ratios in the patient and control groups were 46/70 and 89/132, respectively.

### SNP genotyping

For a search of the 44 SNPs of 13 genes along with HLA-A alleles (listed in Table E2), SNP genotyping was performed by using PCR direct sequencing. Genomic DNA was isolated from human peripheral blood at SRL, Inc (Tokyo, Japan). For direct sequencing, PCR amplification was conducted with AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, Calif) for 35 cycles at 94°C for 1 minute and annealing at 60°C for 1 minute and 72°C for 1 minute on a commercial PCR machine (GeneAmp; PerkinElmer, Applied Biosystems). The PCR products were reacted with BigDye Terminator version 3.1 (Applied Biosystems), and sequence reactions were resolved on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

To obtain more detailed information of genetic variants in the *TLR3* and *PTGER3* regions, we genotyped 116 patients with SJS/TEN and 221 healthy control subjects for 42 SNPs by using the DigiTag2 and TaqMan SNP genotyping assays (Applied Biosystems). In the DigiTag2 assay we designed multiplex PCR primers for each of the 32 SNP sites. Multiplex PCR was performed in 10  $\mu$ L of Multiplex PCR buffer containing 25 ng of genomic DNA, 25 nmol/L of each multiplex primer mix, 200  $\mu$ mol/L of each deoxyribonucleoside triphosphate, 2.25 mmol/L MgCl<sub>2</sub>, and 0.4 U of KAPA2G Fast HotStart DNA polymerase (Kapa Biosystems, Mowbray, South Africa). Cycling was performed at 95°C for 3 minutes, followed by 40 cycles of 95°C for 15 seconds and 68°C for 2 minutes. We used 36 SNPs covering a greater than 95% call rate for further analyses (Table E1). In the TaqMan SNP genotyping assay PCR amplification was performed in a 5- $\mu$ L reaction mixture containing 1  $\mu$ L of genomic DNA, 2.5  $\mu$ L of Absolute QPCR ROX Mix (Thermo Fisher Scientific, Inc, Waltham, Mass), and  $\times 40$  TaqMan SNP Genotyping Assay probe (Applied Biosystems) for each SNP. The QPCR thermal cycling program was 95°C for 15 minutes, followed by 40 cycles of 95°C for

15 seconds and 60°C for 1 minute. All samples subjected to the DigiTag2 assay and the TaqMan SNP genotyping assay were found to have a greater than 95% call rate. The 7 SNPs of *TLR3* and 6 SNPs of *PTGER3*, which we have reported previously, were examined by using PCR direct sequencing, as described above. The primers and probes used in this study are shown in Table E3.

### HLA-A genotyping

For HLA-A genotyping, we performed PCR amplification followed by hybridization with sequence-specific oligonucleotide probes (PCR-SSO) by using commercial bead-based typing kits (WAK Flow; Wakunaga, Hiroshima, Japan).

### Statistical analysis

A scan for epistatic interactions in data for multiple loci is associated with serious problems, such as computational burden and high dimensionality. The former restricts potential algorithms to those that are simple and fast, whereas the latter is a theoretic issue with no efficient and universal solution, being known as the “ $p > n$  or  $p \gg n$  problem” or the “curse of dimensionality,” causing standard methods of multivariate regression to break down and prohibitive conservation of alternate methods involving multiple univariate regression caused by necessary corrections of the heavy multiplicity. On the other hand, eclectic methods based on SNP filtering by *P* value could potentially miss interactions with no or only weak marginal effects. Instead of these current methods, we have therefore proposed the use of a model selection strategy for interaction analysis of high-dimensional data. Our new software, EPISIS, implements SIS (Fan and Lv 2008), followed by some iterative steps, which can be roughly regarded as a sophisticated analog of the classical forward-backward stepwise procedure suited to ultra-high-dimensional regression models. SIS has 2 major parts: screening and variable selection. In the screening part candidates are selected on the basis of feature ranking, and then subsequent variable selection from these candidates is carried out for interactions and main effects. As a variable selection algorithm for penalized regression, we use SCAD. We also use LASSO for comparison.

Statistical significance of the association with each SNP was assessed by using the Fisher exact test on  $2 \times 2$  contingency tables. Haploview software (version 4.2) was used to infer the LD structure of specific genomic regions, to perform haplotype association testing, and to permute the data pertaining to their association.

### Mice

BALB/c mice were purchased from CLEA (Tokyo, Japan) and used at 6 to 12 weeks of age for sensitization. *TLR3* KO and *PTGER3* KO mice were generated, as described previously, and back-crossed for more than 7 generations to BALB/c mice. *TLR3/PTGER3* DKO mice were generated by interbreeding of *TLR3* KO and *PTGER3* KO mice at Kyoto Prefectural University of Medicine. They were subjected to EAC at 9 to 15 weeks of age, with age-matched, wild-type BALB/c mice as control animals. The mice were maintained on a 12-hour/12-hour light/dark cycle under specific pathogen-free conditions. All experimental procedures were approved by the Committee on Animal Research of Kyoto Prefectural University of Medicine, Kyoto, Japan. All studies were performed in accordance with the Association for Research in Vision and Ophthalmology’s “Statement for the use of animals in ophthalmic and vision research.”

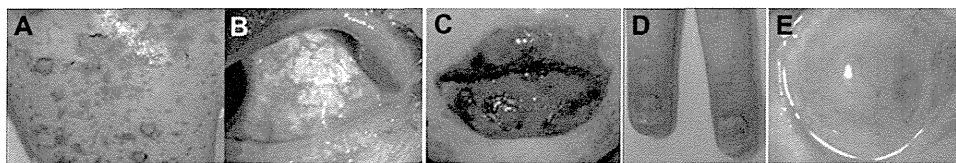
### Eosinophil infiltration in a murine model of EAC

The experiments were conducted by using a protocol approved by the Institutional Animal Care and Use Committee of Kyoto Prefectural University of Medicine. Short RW was purchased from Polysciences, Inc (Warrington, Pa), and aluminum hydroxide (alum) was from Sigma-Aldrich (St Louis, Mo). The mice were immunized with an intracutaneous injection of RW adsorbed on alum (200  $\mu$ g of RW and 2.6 mg of alum) into the left hind footpad on day 0. On day 7, they received an intraperitoneal injection of RW adsorbed on alum, and on day 18, their eyes were challenged with RW in PBS (500  $\mu$ g in 5  $\mu$ L per

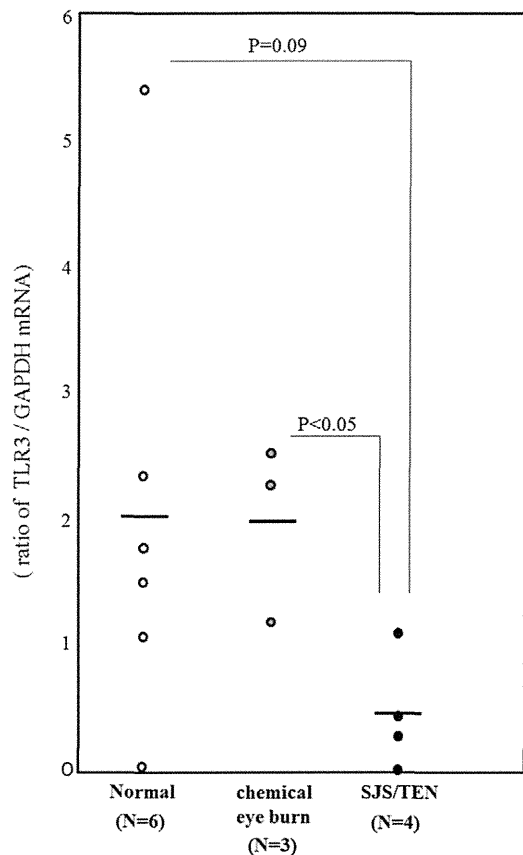
eye) or with PBS alone (5  $\mu$ L per eye). Their eyes, including the conjunctiva, were harvested 24 hours after the last challenge, fixed in 10% neutral-buffered formalin, and embedded in paraffin blocks for histologic analysis. Vertical 6- $\mu$ m-thick sections were mounted on microscope slides, deparaffinized, and stained with Luna stain, which identifies erythrocytes and eosinophil granules.

Using an entire section from the central portion of the eye, including the pupil and optic nerve head, we counted infiltrating eosinophils in the lamina propria mucosae of the tarsal conjunctiva. Cell counts were expressed as the number of infiltrating eosinophils per unit area (0.1 mm<sup>2</sup>) measured with image software (Scion Corp, Frederick, Md).

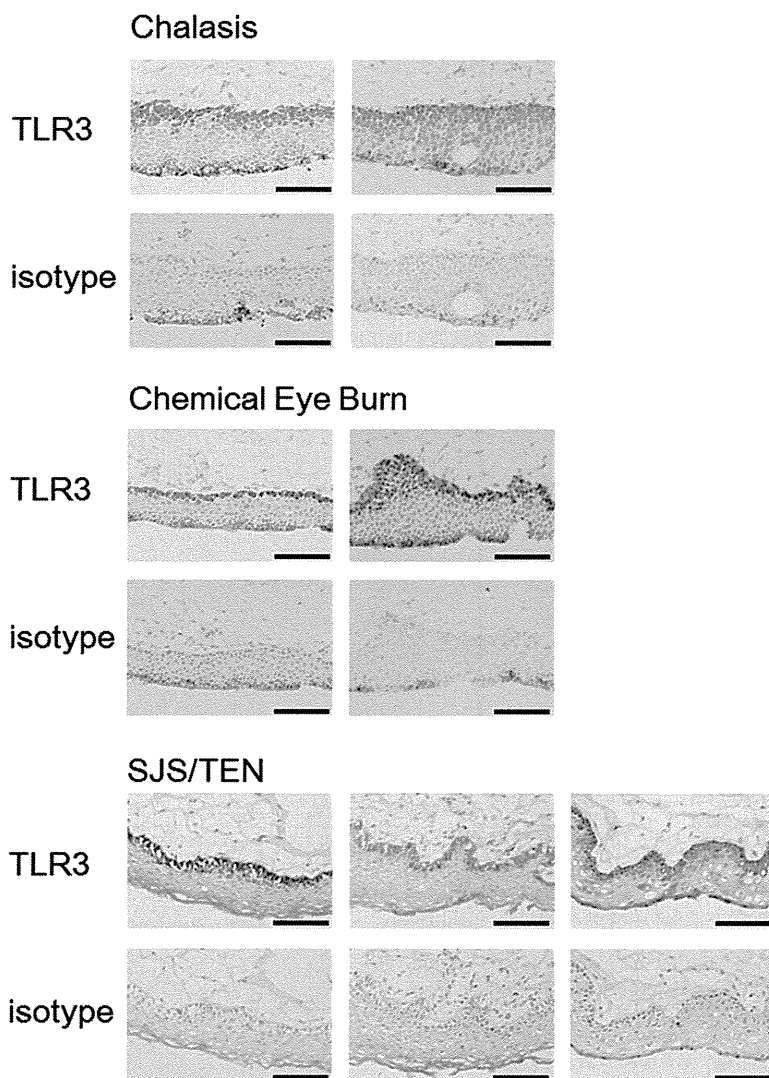




**FIG E1.** Features of patients with SJS/TEN with ocular complications. **A**, Vesiculobullous lesions of the skin in the acute stage. **B**, Severe conjunctivitis in the acute stage. **C**, Oral involvement, including blisters, erosions, and bleeding of the mouth and lips in the acute stage. **D**, Paronychia in the acute stage. **E**, Conjunctival invasion into the cornea in the chronic stage.



**FIG E2.** Expression of *TLR3* mRNA in conjunctival tissues from patients with SJS/TEN and chemical eye burn and the control subjects. Total RNA was isolated from conjunctival tissue sections by using the RNeasy mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The RT reaction was with the SuperScript preamplification kit (Invitrogen, Carlsbad, Calif). Quantitative RT-PCR was on an ABI-prism 7700 instrument (Applied Biosystems). The probes for human *TLR3* and human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were from Applied Biosystems. The results were analyzed with sequence detection software (Applied Biosystems). The quantification data were normalized to the expression of the housekeeping gene *GAPDH*.



**FIG E3.** Immunohistologic analysis of *TLR3* in conjunctival epithelium of patients with SJS/TEN and chemical eye burn and control subjects. For TLR3 staining, we used rabbit polyclonal antibody to TLR3 (Abcam, Cambridge, Mass). The secondary antibody (Biotin-SP-conjugated AffiniPure F[ab']<sub>2</sub> fragment donkey anti-rabbit IgG [H+L], 1:500 dilution; Jackson ImmunoResearch, Baltimore, Md) was applied for 30 minutes. The VECTASTAIN ABC reagent (Vector Laboratories, Burlingame, Calif) was used for increased sensitivity with peroxidase substrate solution (DAB substrate kit; Vector Laboratories) as a chromogenic substrate. Each *scale bar* represents 100  $\mu$ m.

**TABLE E1.** Primers and probes used in SNP analysis of *TLR3* and *PTGER3*

Gene	rsID	Genotyping	Primer sequence or context sequence (VIC/FAM)
<i>TLR3</i>	rs4861699	DigiTag2	AACTTAAAGAAGTGAAAGACTTGAACACTGAAAACATAAA AGGTATTTAAACTAATTTGAGTGGATTTTTGTGTATGGTGTA
	rs6822014	DigiTag2	ATAACTTGATGAGCTTGAAGACAAGTATACTTCTGTGAAA GGCATAACATAAATGGAATATTATTCTACCTTACAAAA
	rs4862632	DigiTag2	GCTTGATCTGCAAACATAAGTGACATACGCAAACATAATAA ACCATTTGTTTAGGTTTATAATATATTCATCGCATTACATA
	rs5743305	DigiTag2	ACTCACTTTTTTTCATTACAGATGTGCTATGATCTATTATA CAAGGCGCTCACAGAGAAGAAATCTTTGAATATTAGTGAA
	rs11732384	DigiTag2	AAATTATTCCAGGTAAGTGTGAGGTAATAAAATCACCTTA GAGGGGTACATCTCACCTAAGCAAGGAGAATGTATTGTA
	rs7657186	DigiTag2	AGGCCAATACCACATTGTTCCGATTACTTTAGCTTTACAA CAACCACCACACTTTTAAACGACCGAATCTCATAA
	rs6552950	DigiTag2	TGTTGCACCACCAACTTCCCTGACAACATTGGTA ATTAACCTAGGAGAGGTCACACACCTTCACATAGAAGCTAA
	rs5743312	DigiTag2	TCCTATGAAGCAGAGTCATTATCACGCCCATTTGAAA AGTTGTCATCGAATCAAATTAAGAGGTAAGAAGTAAGGTA
	rs7668666	DigiTag2	AGTGCCTTAACAGTGTGAATTCAGTACAGTAAGAATTTAA GAGGGTACGTGTCTGGATCATGAAGACAGACTA
	rs3775292	DigiTag2	GAGAAAATCCGGTGGAAAGACGAGAGGGAGAGCTA GACAGATTCCGAATGCTTGTGTTGCTAATTCCAAACATA
	rs3775291	DigiTag2	AAGCAATATGTTACAGGATTGATAAACCTGAAATACTTA TTGGCTATGTTGTTGCTTAGATCCAGAATGGTCAA
	rs10025405	DigiTag2	AGCTATTCAGGCTATTTCCTAATAATCAGATTCTCTTTATTGTA GTTTCAATGGGTATAATGCTATTTCCTTTGTAAGAGTA
	rs3775296	Direct sequencing	TTACCTTCTGCTTGACAAAGGG TGCATTTGAAAGCCATCTGC
	rs3775295	Direct sequencing	TCACATGGCTTATCAAACACACAG
	rs3775294	Direct sequencing	CATTGCTTCTCTCAGATGCC
	rs3775293		CAGTCTTTACTCCATCTCCGC
	rs3775292		CCAAGGCTCTGGTAAGGGTG
	rs3775291	Direct sequencing	TGGCTAAAATGTTGGAGCA
	rs3775290	Direct sequencing	GAAGAGGCTGGAATGGTGAA
rs11803673	DigiTag2		GAAACACATCCTGGAGTCATTCTGATGGGATTGCTA GGACAGGGAGAGAGGGACGGAAGAAAAAATAATCAA
<i>PTGER3</i>	rs7555865	DigiTag2	GAAACACATCCTGGAGTCATTCTGATGGGATTGCTA GGACAGGGAGAGAGGGACGGAAGAAAAAATAATCAA
	rs1327453	DigiTag2	AGTTACCATACACAAATGGAACACACATAACATGATGA TCAGTTCAACCTAACTGGTCTTCTACCCAGTCATTCATAA
	rs4320735	DigiTag2	AGACTTGAGTCTAGAATTGCTTTGTTGAGAGAGATAGGTA TGCAAGTGACAGAAATCAAAGTCAATTTAGCTTAGGTATA
	rs2182324	DigiTag2	ATAATGCCATCTTGCTACTATGTAGAGCAGAACTTTCAA CCATAAGGGTTTAAAATCTTTATCACTTCACTGATGTATA
	rs10443262	DigiTag2	GACAGCCATCACAGGATCAAAGACCTAGGAAGGAA TAGTCTCATTGACTCCATGCCCATATGCTGGACA
	rs2421805	DigiTag2	AGATACGTAATGAAAGTGGTCTCTTGTGGTCTCTTCTTA ACACAGAGAGGCCTAACACTGAAAACCAATGACATAAAAA
	rs2225025	DigiTag2	GGAAGGTCACCTACAAAGGGAACCTATCAAGCTAA TTTCCTTTCCATATTTAGCACTCCCTTAATGACTGTGCAA
	rs1409981	DigiTag2	TAGGGGATGTGAGAAGGAGGGTCTGAAGATGAAA AAGCTGCCTTTACCCATACCTATTTGAGTTACTCAGAAA
	rs6667891	DigiTag2	TAGGGGATGTGAGAAGGAGGGTCTGAAGATGAAA AAGCTGCCTTTACCCATACCTATTTGAGTTACTCAGAAA
	rs4147115	DigiTag2	CCTGAAACTCCATATTTTACAACCTCACCTCTGTGATTTTTCA ATCCTAACAGTTACTTTGTCTTATCAGTTTTAGCACTTA
	rs4650093	DigiTag2	ATCGAAACTCCATATTTTACAACCTCACCTCTGTGATTTTTCA ATCCTAACAGTTACTTTGTCTTATCAGTTTTAGCACTTA
	rs17131478	DigiTag2	CCTGAAACTCCATATTTTACAACCTCACCTCTGTGATTTTTCA ATCCTAACAGTTACTTTGTCTTATCAGTTTTAGCACTTA
	rs17131479	DigiTag2	CCTGAAACTCCATATTTTACAACCTCACCTCTGTGATTTTTCA ATCCTAACAGTTACTTTGTCTTATCAGTTTTAGCACTTA
	rs7521005	DigiTag2	ATGAACCTGACATATGAACCTTAAAAGCTAGAATTTAACTAAA CTTTTCTCAGATCCTCATCTCCTCATGCAATATGCCATAA
	rs12039590	DigiTag2	AGAGTGTGGACTATCACTTGTCAAATATTTGAGAAAATA AGGTATGTGAATCCTTATAACAGTCTTAAGGAGTAGACGTTA

(Continued)

TABLE E1. (Continued)

Gene	rsID	Genotyping	Primer sequence or context sequence (VIC/FAM)
	rs7541092	DigiTag2	AGGGAGATACAAATCAAACAAAAACATGTTGAAGTCAATA CATCTACAGGTCAAGAAATGCCTAGGAATGCCAGAAAA
	rs2068652	DigiTag2	AGGGAGATACAAATCAAACAAAAACATGTTGAAGTCAATA CATCTACAGGTCAAGAAATGCCTAGGAATGCCAGAAAA
	rs17131485	DigiTag2	CTGTGGAGAAGAATCTACCACCTTGATCTGGAGTTGTA GTTTGATTGTATCTCCCTAAAATATCATCAGTTCTTCAA
	rs11209710	DigiTag2	AGTATTGAAGAGTCTAATACTGAGTCATTGAAGGATATAGTA ATGCTTGAAGAATGCTCCAAGAAATGGACTATTCTCATATA
	rs1359835	DigiTag2	AGTATTGAAGAGTCTAATACTGAGTCATTGAAGGATATAGTA ATGCTTGAAGAATGCTCCAAGAAATGGACTATTCTCATATA
	rs1327464	DigiTag2	CTGCTTTGTAACCTGGGCTGGAGAGGTTTATCCAA CTTCAATACTTCACTAGACTTTCTGGATGCAATGACTGTA
	rs12048245	TaqMan	TCTAGGAGATTCTGAGACAGGTGTT[C/T] GCTTCAAAGGAAAAGCTTTTGAAA
	rs10889897	TaqMan	AGGAAAGGCATCAAAGGAATTGCAC[A/G] GGGTAGGAAAGTACAACGGATATTC
	rs1409161	TaqMan	CTTAATTTTAGGCTTCTGGTCTCCA[A/G] AACTGGAAGAAAATAGTTTGGGTA
	rs12123324	TaqMan	GAAGCTCCTCAAGTGTAGAGTTCA[C/T] AAGATGTTGGGTAACGTACAGTTT
	rs6670616	TaqMan	TAAAGACGAATAAATACAGCTGTGT[A/T] TTGATTCGCACCTTTCCTATGACA
	rs17090700	TaqMan	CATGACATTTGGGATTAAGTTCTGC[A/C] TTTTAGAGTACTCATTCAATGAAGT
	rs909848	TaqMan	TCATTAATAGTTCTTTCTGCTCACC[C/T] ACACTAGCTCACTAATTTATCCCA
	rs11209733	TaqMan	AATTTGTAAGTGTATATTAGCATTAA[C/G] TGTAGTCATCTACAGGAGTATAGA
	rs17131450	Direct sequencing	TTTATGCAGCTTTCGGTCA CCCCTCCAGGCTGATAACTC
	rs5702	Direct sequencing	CAAGTAGCAGTTGGCAGCAA TGCAATCAGACAGGCAAGAG
	rs1325949	Direct sequencing	AATTGCAAGTCCAGCTCAGG AGGCCTCAGGGAGCTTTTAC
	rs7543182	Direct sequencing	TGTGAGGCAAGAACCAGACA AGGACCTGGGAGGGAAGATA
	rs7555874	Direct sequencing	AAGCCAGCAAAGGACAAGAA TGTTGTGTGTCTGCCAGGTT
	rs4147114	Direct sequencing	TGCTGGAAGCTCATGGTCTA TGCATGGTTCGTCTAACCTTAT