Based on LD structure, we selected rs2304256 as a tag SNP of the other two SNPs, because it is a non-synonymous SNP resulting in a Val → Phe substitution, suggesting putative functional importance. The functional significance of *TYK2* in autoimmune disorders is well documented in both mice and humans. *Tyk2−/−* mice display reduced responses to type I IFN (IFN-$\alpha/\beta$) and IL12, leading to impaired STAT3 activation [18]. TYK2 deficiency is also observed in humans with hyper-IgE syndrome (HIES) who show almost complete defects in type I IFN signaling and other cytokine signalings [19, 20]. Thus, the fact that SLE patients often show abnormal type I IFN signaling is suggestive of *TYK2*'s involvement in the pathogenesis of human SLE.

TYK2's key role in type I IFN signaling has been addressed by many researchers so far. Upon the binding of IFN-$\alpha$ to IFNAR, TYK2 physically interacts with IFNAR1/IFNAR2 heterocomplex together with JAK1, activating STAT1 and STAT2 complexed with IRF9 to form the trimeric ISGF3. ISGF3 enters the nucleus and binds to a consensus DNA sequence called the IFN-stimulated response element (ISRE) to induce expression of type I IFN genes (e.g., IFN-$\alpha$ and IFN-$\beta$), stimulating further transcription of type I IFN-mediated genes [10]. TYK2, which is reportedly essential for stable cell surface expression of IFNAR1 [21], possesses a C-terminal tyrosine kinase (TK) catalytic domain, a central kinase-like (KL) domain, and a large N-terminal region that is subdivided into five JAK homology regions (JH7 to JH3) [22]. The position of rs2304256 Val → Phe substitution is within the JH4 region. The functional relevance of this SNP remains unclear, but JH regions are known to be important for TYK2 binding to IFNAR. In particular, the JH5-4-3 region is known to contribute to stable binding [23]. From these insights, rs2304256 may be related to an abnormal downstream modulation of the type I IFN signaling pathway that is often featured in SLE. Especially in the Caucasian population, a possibility that *TYK2* SNPs are related to serological and clinical manifestations of SLE, such as type I IFN levels, and the SLEDAI severity score needs to be addressed in future studies.

In sum, our data indicate that none of the SLE-associated *TYK2* SNPs in Caucasians are a genetic risk factor in the Japanese. Homozygocity for the minor A allele of rs2304256 occurs slightly more frequently in Japanese SLEs compared to healthy controls; however, a statistically significant association was not observed. Because of *TYK2*'s functional importance, it may be a crucial risk factor for SLE in Caucasians, but a minor factor in Asians. Examining ethnic differences in disease-associated genes is our great interest. An additional comprehensive genotyping of *TYK2* SNPs in other Asian ethnic groups than Japanese will provide us clues to understanding this.

**Acknowledgments** We acknowledge Dr. Osamu Shirakawa (Kinki University School of Medicine) who kindly provided the second set of healthy control samples. We thank all the patients and healthy donors, as well as the laboratory workers who were devoted to collecting and analyzing the samples, especially Mr. Kazuhisa Kishimoto (Kobe University Graduate School of Medicine). The work was supported by the 21st Century COE Program and research grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and the Science and Technology Foundation of Japan.

**Conflict of interest statement** The authors declare no conflicts of interest.

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# A Genome-Wide Association Analysis Identified a Novel Susceptible Locus for Pathological Myopia at 11q24.1

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## Abstract

Myopia is one of the most common ocular disorders worldwide. Pathological myopia, also called high myopia, comprises 1% to 5% of the general population and is one of the leading causes of legal blindness in developed countries. To identify genetic determinants associated with pathological myopia in Japanese, we conducted a genome-wide association study, analyzing 411,777 SNPs with 830 cases and 1,911 general population controls in a two-stage design (297 cases and 934 controls in the first stage and 533 cases and 977 controls in the second stage). We selected 22 SNPs that showed *P*-values smaller than $10^{-4}$ in the first stage and tested them for association in the second stage. The meta-analysis combining the first and second stages identified an SNP, rs577948, at chromosome 11q24.1, which was associated with the disease ($P = 2.22 \times 10^{-7}$ and OR of 1.37 with 95% confidence interval: 1.21–1.54). Two genes, *BLID* and *LOC399959*, were identified within a 200-kb DNA encompassing rs577948. RT–PCR analysis demonstrated that both genes were expressed in human retinal tissue. Our results strongly suggest that the region at 11q24.1 is a novel susceptibility locus for pathological myopia in Japanese.

**Citation:** Nakanishi H, Yamada R, Gotoh N, Hayashi H, Yamashiro K, et al. (2009) A Genome-Wide Association Analysis Identified a Novel Susceptible Locus for Pathological Myopia at 11q24.1. PLoS Genet 5(9): e1000660. doi:10.1371/journal.pgen.1000660

**Editor:** Takashi Gojobori, National Institute of Genetics, Japan

**Received** May 26, 2009; **Accepted** August 24, 2009; **Published** September 25, 2009



**Funding:** The study was supported in part by grants-in-aid for scientific research (19390442 and 27091294) from the Japan Society for the Promotion of Science, Tokyo, Japan; by Okawa Foundation for Information and Telecommunications; and by the Japanese National Society for the Prevention of Blindness. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

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## Introduction

Myopia is a refractive error (http://en.wikipedia.org/wiki/Refractive_error) of the eye in which parallel rays of light focus in a plane anterior to the retina resulting in blurred vision. Myopia is one of the most common ocular disorders worldwide, and is in much higher prevalence in Asians than in Caucasians. Recent population-based surveys in the elderly reported that the prevalence of myopia was approximately 25% in the Caucasian populations [1] and 40% in the East Asian (Chinese and Japanese) populations [2,3].

Myopia is divided into two distinct subsets, namely, common and pathological myopia. Pathological myopia, also called high myopia, is distinguished from common myopia, also called low/moderate myopia, by excessive increase in axial length of the eyeball, which is the most important contributor to the myopic refraction [4,5]. The axial length of the eyeball in adults is approximately 24 mm, and its elongation by 1 mm without other compensatory changes results in a myopic shift of −2.5 to −3.0 diopters (D). It has been shown that distribution of the axial lengths of the adult myopic population is bimodal [6], and the subgroup with elongated axial length in the bimodal distribution corresponds to pathological myopia. This group comprises 1% to 5% of the population [3,7], and is commonly defined by axial length greater than 26.0 mm which is equivalent to refractive errors greater than −6 D [8].

The excessive elongation of the eyeball causes mechanical strain with subsequent degenerative changes of the retina, choroid, and sclera. The degenerative changes at the posterior pole of the eye such as chorioretinal atrophy or posterior staphyloma are clinically important and unique to pathological myopia [9]. These unique degenerative changes at the posterior pole result in uncorrectable visual impairment due to decreased central vision and make pathological myopia one of the leading causes of legal blindness in developed countries [10–13].

It has been reported that not only environmental factors, such as near work and higher education, but also genetic factors contribute to the development of myopia, in particular, of pathological myopia [14]. Previous twin studies reported that the estimated heritability of refractive error and axial length is up to 0.90 [15,16], although that might be overestimated due to common environmental effects [17]. Multiple family-based whole genome linkage analyses of myopia reported at least 16 susceptible chromosomal loci (MYP1–16 in OMIM database; 10 loci for

## Author Summary

Myopia is one of the most common ocular disorders with elongation of axis of the eyeball. Pathological myopia or high myopia, a subset of myopia which is characterized with excessive axial elongation and degenerative changes of the eye, is a leading cause of visual impairment. Since genetic factors play significant roles in its development, identification of genetic determinants is an urgent and important issue. Although family-based linkage analyses have isolated at least 16 susceptible chromosomal loci for pathological or common myopia, no gene responsible for the disease has been identified. We conducted the first genome-wide case/control association study of pathological myopia in a two-stage design using 411,777 markers with 830 Japanese patients and 1,911 Japanese controls. We identified a region strongly suggestive for the disease susceptibility at chromosome 11q24.1 containing *BLID* and *LOC399959*. Their expression was confirmed in human retina with RT–PCR. *BLID* encodes an inducer of apoptotic cell death, and apoptosis is known to play an important functional role in pathological myopia. We believe that our study contributes to further dissect the molecular events underlying the development and progression of pathological myopia.


pathological myopia [18–27] and 6 for common myopia [28–30]). Among them, at least 8 chromosomal loci, such as 12q21–23 (MYP3), 22q12 (MYP6) and 2q37.1 (MYP12) were successfully validated by at least two independent studies [31,32]. However, no genes responsible for the disease have been identified.

The genome-wide association (GWA) study using single nucleotide polymorphisms (SNPs) as markers is an alternative approach to identify genetic risk factors of common diseases. This approach has been successfully applied to identify genetic risk factors for multigenetic diseases including ophthalmic diseases such as age-related macular degeneration [33,34] and exfoliation syndrome [35]. To identify the genetic risk factors of pathological myopia, we conducted a two-stage GWA-based case/control association analysis using 411,777 markers with 830 Japanese patients and 1,911 Japanese controls (297 cases and 934 controls in the first stage, and 533 cases and 977 controls in the second stage).

## Results

### Characterization of the patients with pathological myopia

A total of 839 pathological myopic patients with axial length greater than 26.0 mm in both eyes were enrolled in the current study. In order to maximize the detection power, patients with axial length greater than 28.0 mm in both eyes were enrolled in the first stage of genome scan. No other clinical features were accounted for the assignment of patients to either stage. 824 out of 839 patients (98.2%) had degenerative changes specific to pathological myopia. Other features of cases and controls who passed quality control procedures of genotyping results (see Materials and Methods) were summarized in Table 1.

### Genome-wide association analysis

For the first stage, we scanned the genome of 302 cases using the Illumina HumanHap550 BeadChip, which launches 561,466 relatively frequent SNPs (minor allele frequency >0.05) distributed across the human genome at an average interval of 6.5 kilobases (kb). Five cases and 149,689 SNPs were excluded due to quality control criteria (see details in Materials and Methods) and genotyping results of 411,777 SNPs in autosomes for 297 cases were used for the statistical analysis. They were compared with 934 controls from the JSNP database [36] for association with phenotype using $\chi^2$ test for trend. Genomic Control (GC) method [37] revealed only a slight inflation of the test statistics (GC parameter $\lambda = 1.068$). We identified 29 SNPs in 22 chromosomal regions with *P*-value adjusted by GC being smaller than $10^{-4}$ (Figure 1 and Table S1). Among them, seven SNPs at chromosome 8p12 were in strong linkage disequilibrium (LD) and likewise two SNPs at chromosome 10q22.2 (pair-wise $D' > 0.95$ and $r^2 > 0.9$). Thus, we selected one representing SNP from each region and tested 22 SNPs in the second stage.

**Table 1.** Characteristics of the study population used in the study[a].

| Cases/Controls | Category | Subcategory | First stage | Second stage |
|---|---|---|---|---|
| **Cases: Patients with pathological myopia** | Number | | 297 | 533 |
| | Age (years) | | 58.8±13.2 | 59.0±14.3 |
| | Gender | Male | 93 | 171 |
| | | Female | 204 | 362 |
| | Axial length (mm) | Right eyes | 29.97±1.36 | 29.04±1.97 |
| | | Left eyes | 29.84±1.37 | 28.91±1.89 |
| | Refraction of the phakic eyes (Diopter)[b] | Right eyes | −14.94±4.04 | −12.40±4.48 |
| | | Left eyes | −14.64±3.98 | −12.07±4.72 |
| **Controls: general Japanese population** | Number | | 934 | 977 |
| | Age (years) | | NA | 48.3±16.3 |
| | Gender | Male | NA | 497 |
| | | Female | NA | 480 |

The ± sign is a standard deviation.
[a]The study population after quality control procedures.
[b]For the calculations of refraction, 177 eyes (29.8%) in the first stage and 303 eyes (28.4%) in the second stage that had undergone cataract surgery or corneal refractive surgery were excluded.
doi:10.1371/journal.pgen.1000660.t001

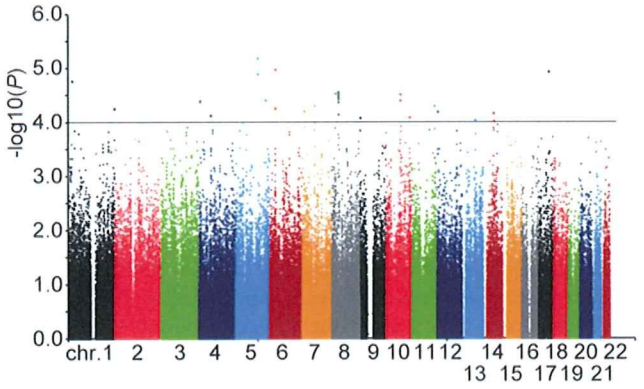


**Figure 1. Manhattan plot of the first stage results for pathological myopia.** Adjusted *P*-values obtained by the trend $\chi^2$ test for 411,777 SNPs on autosomes in 297 pathological myopic cases and 934 general population controls are plotted in $-\log_{10}$ scale according to their chromosome location.
doi:10.1371/journal.pgen.1000660.g001

For the second stage analysis, 537 cases and 980 population controls were genotyped by Taqman method. Among them, four cases and three controls were excluded due to low call rates (<90%). Genotyping success rates of the 22 SNP markers in the remaining 1,510 samples were greater than 96.8%. The genotype counts of the first and second stages were combined for meta-analysis. One SNP, rs577948, showed a strongly suggestive association ($P=2.22\times10^{-7}$) (Table 2) in the meta-analysis whereas the remaining 21 SNPs were not significant ($P>10^{-5}$) (Table S1).

## Evaluation of the region with rs577948

The SNP rs577948 which showed $P=2.22\times10^{-7}$ by meta-analysis with OR of 1.37 (95% confidence interval (CI): 1.21–1.54) for the risk allele (nominal $P=2.80\times10^{-5}$ and $P=1.42\times10^{-3}$ in the first and second stages, respectively) (Table 2) was located at chromosome 11q24.1 (Figure 2A). Using the results of the first stage, an LD block which extended a 55-kb region containing rs577948 was generated. Six additional SNP markers within the block were included in the genome scan chip (Figure 2B). Among them, we selected three markers with adjusted *P*-value smaller than 0.01 in the first stage for further genotyping by Taqman method with DNAs used for the second stage. Weaker associations than that of rs577948 were obtained for these three markers by meta-analysis (Table 2). As shown in Figure 2B, two genes were located in a 200-kb region containing rs577948. *BLID* is a cell death inducer containing BH3-like motif [38], which is located approximately 44-kb upstream of rs577948. The other gene, *LOC399959*, is a hypothetical non-coding RNA [39] which encompassed 114-kb DNA in the region, and rs577948 is located in its second intron.

## Expression of the *BLID* and *LOC399959*

*BLID* is known as a cell-death inducer expressed in cytoplasm, in mitochondria at lower abundance, and in various human cancer cells from different tissues [38]. *LOC399959* was reported as a hypothetical non-coding RNA with a relatively ubiquitous expression pattern. We assessed the expression of the genes by RT-PCR using cDNAs of human retina and brain and those of HeLa cells as positive control. Expressions of both genes were detected in human retinal tissue as well as in human brain and HeLa cells (Figure 3).

**Table 2.** Association of SNP markers within the linkage disequilibrium block on chromosome 11q24.1 with pathological myopia in Japanese population.

| | | | | Meta-analysis[c] | | First stage (N = 1,231) | | | | Second stage (N = 1,510) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SNP ID | Position[a] | Ref.[b] | Var.[b] | *P*-value | OR (95%CI)[d] | Ref. allele freq. | | Nominal *P* | OR (95%CI)[d] | Ref. allele freq. | | Nominal *P* | OR (95%CI)[d] |
| | | | | | | Case (N = 297) | Control (N = 934) | | | Case (N = 533) | Control (N = 977) | | |
| rs577948 | 121535400 | A | G* | $2.22\times10^{-7}$ | 1.37 (1.21–1.54) | 0.40 | 0.50 | $2.80\times10^{-5}$ | 1.50 (1.24–1.81) | 0.42 | 0.48 | $1.42\times10^{-3}$ | 1.29 (1.11–1.50) |
| rs11218544 | 121544262 | T* | G | $5.48\times10^{-6}$ | 1.33 (1.18–1.51) | 0.70 | 0.61 | $7.90\times10^{-5}$ | 1.50 (1.23–1.83) | 0.66 | 0.61 | $8.94\times10^{-3}$ | 1.24 (1.06–1.44) |
| rs10892819 | 121579254 | T | G* | 0.04 | 1.15 (1.01–1.31) | 0.69 | 0.75 | $2.98\times10^{-3}$ | 1.36 (1.11–1.67) | 0.72 | 0.73 | 0.74 | 1.03 (0.87–1.22) |
| rs11218553 | 121590345 | A | G* | $8.28\times10^{-3}$ | 1.18 (1.04–1.34) | 0.60 | 0.67 | $1.77\times10^{-3}$ | 1.36 (1.12–1.65) | 0.66 | 0.68 | 0.39 | 1.07 (0.91–1.26) |

[a]The position of markers on chromosome 11 refers to NCBI Build 36.1.
[b]Ref. and Var. are the reference and variant nucleotides, respectively, that are defined on the reference sequence of NCBI Build 36.1.
[c]Statistical results using the Mantel-Haenzel method as a fixed-effect model were shown.
[d]Odds ratios (ORs) were calculated for the causative allele (indicated with an asterisk).
doi:10.1371/journal.pgen.1000660.t002

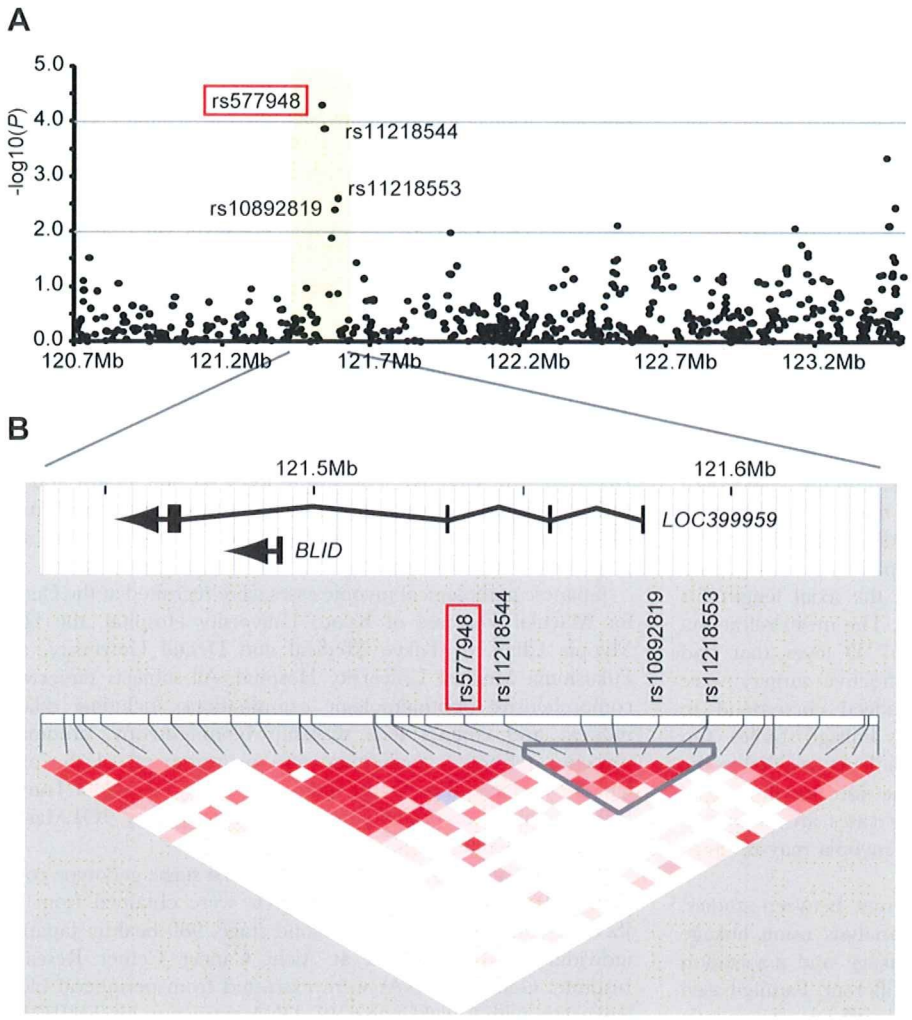


**Figure 2. Results of genome scan at 11q24.1 locus containing the *BLID* and *LOC399959* genes.** (A) Adjusted *P*-values on $-\log_{10}$ scale for SNPs examined for their association by the trend $\chi^2$ test. (B) Structures, orientations and locations of the *BLID* and *LOC399959* genes on NCBI Reference Sequence Build 36.1, together with pair-wise LD estimates of the SNP markers located within a 200-kb region encompassing the rs577948 marker (red box). Three additional SNP markers (rs11218544, rs11218553, and rs10892819), that showed adjusted *P*-value$<10^{-2}$ in the first stage, are also indicated.
doi:10.1371/journal.pgen.1000660.g002

## Discussion

Myopic refraction and axial length are reported to be a complex trait under polygenic control in which contribution of each gene is relatively small [40]. In the current study, two-stage GWA analysis identified a region at chromosome 11q24.1, in which rs577948 showed strongly suggestive $P=2.22\times10^{-7}$ with OR of 1.37 (95% CI: 1.21–1.54) for the allele G.

Our GWA study identified only one strongly-suggestive locus. This may principally be due to the sample size of our study not being adequate. Recent genetic studies of complex traits with higher prevalence enroll much larger number of samples. In contrast, recruitment of patients with pathological myopia is difficult due to its lower prevalence, particularly those with degenerative changes (namely degenerative myopia). In order to improve insufficient detection power, we assigned pathological myopia patients with longer axis (greater than 28.0 mm) to the first stage. This strategy might be the reason we were successful in identifying the candidate region with relatively small number of cases.

Insufficiency of detection power due to a limitation in sample number may be a reason for difference between the findings of preceding linkage studies and ours. OMIM database lists 10 MYP regions (MYP1–5, 11–13, 15 and 16) for pathological myopia [18–27]

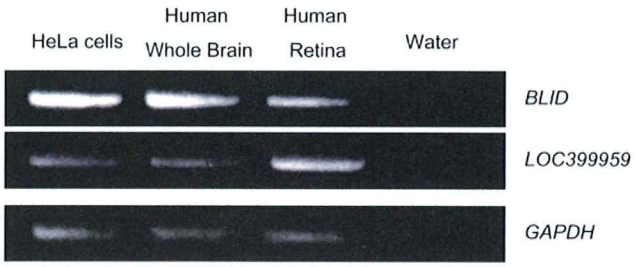


**Figure 3. Expression of the *BLID* and *LOC399959* genes in the human retina.** RT–PCR analyses of *BLID* and *LOC399959* expression in HeLa cells, the human Brain and the human retina. *GAPDH* was used as an internal control for cDNA quantification.
doi:10.1371/journal.pgen.1000660.g003

and 6 MYP regions (MYP6–10 and 14) for common myopia [28–30]. None of these 16 MYPs are on chromosome 11q. Stambolian and colleagues reported heterogeneity LOD score of 1.24 at 11q23 in their linkage study for common myopia in Ashkenazi Jewish descent, which is the closest locus to our region reported to date [29]. Because the linkage signal was not strong and the band 11q23 (chr11, position 110,000 kb to 120,700 kb in the NCBI database) is more than 800 kilobases apart from our LD block in 11q24.1 (chr11, position 121,535 kb to 121,590 kb), whether or not they overlap each other is inconclusive. On the other hand, our study did not identify the associated SNPs in any of MYPs.

Although the insufficiency of detection power may be a reason for difference between our study and the linkage studies, there are other possible reasons. In general, any difference in the study designs could cause heterogeneous results. Firstly, there are two definitions of pathological myopia based on two distinct criteria, namely, the axial length and refractive error. In the current study, we enrolled pathological myopic patients based on the axial length (greater than 26.0 mm in both eyes), and not on the refractive error commonly used in the previous studies (refractive errors greater than −6 D). We focused on patients with vision-threatening degenerative changes [9] and the axial length fits better than refractive error for our purpose. The mean refraction in our myopic patients was −13.14±4.57 D (eyes that had undergone cataract surgery or corneal refractive surgery were excluded from this calculation) which indeed correspond to pathological myopic group in the previous linkage studies. On the other hand, it is not clear whether the patients enrolled in the linkage studies fulfill our criteria because the distribution of axial length and degenerative phenotypes in the cases are unknown. The difference in definition of pathological myopia may result in different susceptibility loci between studies.

Secondly, the methodology used is different between studies, namely, linkage analysis and association analysis using linkage disequilibrium mapping. The results of linkage and association studies of complex genetic traits are often different. Family-based linkage analysis is much more suitable for identifying rare genetic variants with large effects whereas SNP-based GWA analysis is more powerful in detection of relatively common variants with smaller effects in complex diseases [41].

Finally, the difference can also be due to the ethnicities of the samples enrolled. In the current study, all cases and controls were Japanese. Only one genome-wide linkage study has previously been published for pathological myopia in Japanese [42] and the others were for non-Japanese populations.

It would be interesting and important to examine the association of our locus in other ethnicities. Ethnic variations in disease susceptibility genes have been reported in various genetic traits including ophthalmological disorders. One such example is an SNP in the complement factor H gene (rs1061170) which has a large effect size with age-related macular degeneration in Caucasians [33,43,44] but much smaller in East Asian populations due to a remarkably lower risk allele frequency (~35% in Caucasians and ~5% in East Asians) [45]. Another example is exfoliation syndrome and *LOXL1* where the risk allele of rs1048661 is inverted between Icelandic (allele G) and Japanese (allele T) populations [35,46]. Because of a large variation in prevalence of myopia among ethnic groups, a future trans-ethnic investigation of myopia risk genes will be important to dissect genetic backgrounds underlying the etiology of myopia.

Although the susceptibility locus contains *BLID* and *LOC399959*, it seems premature to discuss the involvement of *LOC399959* in myopia since it is a hypothetical non-coding gene. *BLID* plays a proapoptotic role involving the BH3-like domain by inducing a caspase-dependent mitochondrial cell death pathway [38]. Indeed, several animal and pathological studies suggested the functional role of apoptosis in pathological myopia [47,48]. Moreover, a recent genome-wide linkage study followed by a fine-scale association mapping identified a myopia susceptibility gene locus containing the *PARL* gene which inhibits the mitochondrial pathway of apoptosis by interaction with *OPA1* [49]. In this context, *BLID* seems functionally relevant with the pathogenesis of pathological myopia. However, the true functional origin of association in this region has yet to be determined by further detailed investigation along with replication studies to validate our findings.

## Materials and Methods

### Study subjects

All procedures used in this study conformed to the tenets of the Declaration of Helsinki. The Institutional Review Board and the Ethics Committee of each institution approved the protocols used. All the participants were fully informed of the purpose and procedures, and a written consent was obtained from each.

Japanese pathological myopic cases were recruited at the Center for Macular Diseases of Kyoto University Hospital, the High Myopia Clinic of Tokyo Medical and Dental University, and Fukushima Medical University Hospital. All subjects underwent comprehensive ophthalmologic examinations, including dilated indirect and contact lens slit-lamp biomicroscopy, automatic objective refraction evaluation, and measurement of the axial length by applanation A-scan ultrasonography (UD-6000, Tomey, Nagoya, Japan) or partial coherence interferometry (IOLMaster, Carl Zeiss Meditec, Dublin, CA).

As a general population control of the first stage, genotype count data of 934 healthy Japanese subjects were obtained from the JSNP database [36]. For the second stage, 980 healthy Japanese individuals were recruited at Aichi Cancer Center Research Institute. Genomic DNAs were extracted from peripheral blood leukocytes with QuickGene-610L DNA extraction kit (FUJIFILM Co., Tokyo, Japan).

### Genome-wide association analysis

We designed to scan the genome in two stages. A total of 839 patients and 1,914 controls were separated into two groups; 302 cases and 934 controls for the first stage, and 537 cases and 980 controls for the second stage. In order to increase the detection power, patients with longer axis of the eyeball (greater than 28.0 mm) were principally assigned to the first stage.

For the first stage analysis, 561,466 SNPs were genotyped in 302 patients of pathological myopia using Illumina Human-Hap550 chips (Illumina Inc., San Diego, CA). This chip covers approximately 87% of the common genetic variations in the Asian population [50]. Cluster definition for each SNP was performed using Illumina BeadStudio Genotyping Module. A systematic quality control procedure of the genome scan results was applied as follows. Samples were evaluated for data quality first and markers were subsequently excluded. Genetic proximity of sample pairs was evaluated with pi-hat in PLINK [51] and four samples with indication of kinship or sample duplication were excluded. Genotypes in X chromosome were used for checking the precision of the phenotype record, and only one sample was removed due to mismatch in gender. The final sample size of pathological myopia was 297. As a population-based control, genotype count data by the genome scanning of 934 healthy Japanese subjects using the same chip were obtained from the JSNP database [36]. The chip contained 515,154 markers in

autosomes that are common in the cases and controls. We excluded 78 SNPs due to low successful call rate (<95%) in the cases, 1,760 SNPs due to the distortion of Hardy-Weinberg Equilibrium (HWE) in the controls ($P<10^{-3}$ by HWE exact test) and 46,722 monomorphic SNPs. 54,817 SNPs with minor allele frequency less than 0.05 in both cases and controls were also excluded. After these quality control procedures, a total of 411,777 SNPs were used for the statistical analysis. The genotyping call rate was greater than 97.43% (median call rate 99.99%) for DNA sample and 98.21% (median call rate 100%) for SNP marker.

Association between genotypic distribution of each SNP and the disease was examined using a $\chi^2$ test for trend. The OR and the 95% CI were estimated using Woolf's method [52]. Inflation in the test statistics was assessed using the genomic-control method [37]. Haploview [53] software was used to infer the LD in the targeted regions. SNPs with *P*-value adjusted by genomic control being smaller than $10^{-4}$ were selected as candidates for second stage. Among the candidate SNPs, LD indices (D′ and $r^2$) were calculated with Haploview and when multiple SNPs were in strong LD (D′>0.95 and $r^2$>0.9), one representative SNP was chosen to be genotyped in the second stage.

In the second stage, 537 cases and 980 controls were genotyped with the Taqman SNP assay using the ABI PRISM 7700 system (Applied Biosystems, Foster City, CA). The 302 pathological myopic cases in the first stage were also genotyped to validate the concordance between Illumina Infinium assay and Taqman assay. Samples with low successful call rate (<90%) were excluded from the study. Subsequently four cases and three controls were excluded and data of 533 cases and 977 controls were used for the analysis. The concordance rate ranged between 98.68% and 100% for the 22 SNPs. The genotype counts of the first and second stages were combined for meta-analysis using the Mantel-Haenzel method [54] as a fixed-effect model. The OR heterogeneity between the first stage and the second stage was evaluated using Cochran's Q-statistic *P*-value. The data from the second stage were also evaluated for association independently from the first stage.

### Screening for *BLID* and *LOC399959* expression

Human retina cDNAs were obtained from Takara Bio Inc. (Kyoto, Japan). Total RNA of HeLa cells and human whole brain were also obtained from the same manufacturer and cDNAs were synthesized using the First-Strand cDNA Synthesis Kit (GE Healthcare Life Sciences, Piscataway, NJ). Two pairs of oligonucleotides were synthesized for RT-PCR; 5′-TTGGGTTCCAACAAAGAACC-3′ and 5′-CTTTTACAGGGCCTCAGCAG-3′ for *BLID*, and 5′-GGCGACATCAGACAGACAGA-3′ and 5′-AGGACCAGCTGAAAGGAACA-3′ for *LOC399959*. Expression of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was tested for cDNA quantification using 5′-GACAACAGCCTCAAGATCATCA-3′ and 5′-GGTCCACCACTGACACGTTG-3′. PCR reactions were performed under the following condition: initial denaturation at 96°C or 2 minutes, followed by 35 cycles (for *BLID* and *LOC399959*) or 18 cycles (for *GAPDH*) at 96°C for 20 seconds, 60°C for 40 seconds, and polymerization at 72°C for 40 seconds.

## Supporting Information

**Table S1** Summary results for the 29 SNPs significant at the $P<10^{-4}$ level after population stratification adjustment in the first stage of the genome-wide association analysis.
Found at: doi:10.1371/journal.pgen.1000660.s001 (0.06 MB XLS)

## Acknowledgments

We would like to thank Dr. Mineo Ozaki, Dr. Yasuo Kurimoto, Dr. Akio Oishi, Dr. Shoji Kuriyama, and Dr. Shin Yoshitake for their assistance in recruiting patients. We also thank Dr. Mikita Suyama for his expertise in bioinformatics and Mr. Victor Renault and Ms. Akiko Yoshizumi for their technical assistance in data handling.

## Author Contributions

Conceived and designed the experiments: HN NG. Performed the experiments: HN NG HH. Analyzed the data: HN RY NY FM. Contributed reagents/materials/analysis tools: NG HH KY NS KOM MM MS TI KM KT. Wrote the paper: HN RY NY FM. Obtained the funding: FM, NY. Performed statistical analysis: RY.

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# *ARMS2* (*LOC387715*) Variants in Japanese Patients with Exudative Age-related Macular Degeneration and Polypoidal Choroidal Vasculopathy

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• PURPOSE: To determine the characteristics of the polymorphisms in the *ARMS2* gene in Japanese patients with age-related macular degeneration (AMD) and those with polypoidal choroidal vasculopathy (PCV) and in healthy controls, and also to show possible associations of the polymorphisms with the disease.

• DESIGN: Case-control association study.

• METHODS: Fifty-six unrelated Japanese individuals with AMD, 55 with PCV, and 77 controls were studied. The most common polymorphism in the *ARMS2* gene on chromosome 10 was resequenced. Association tests were performed for inferred haplotypes.

• RESULTS: A total of 22 polymorphisms were identified, and 13 were shared with those in White persons with AMD. The sequence of the deletion-and-insertion polymorphism, de1443ins54, a functional polymorphism causing an instability of the messenger ribonucleic acid of ARMS2 in the Japanese, did not differ from that in White persons. Among the polymorphisms seen in the White population, rs10490923 (R3H) as well as 7 other polymorphisms were not observed in the Japanese. One haplotype, which contained the T allele of the rs10490924 (A69S) and the variant of de1443ins54 polymorphism, had an odds ratio of 3.14 ($P = 7.8 \times 10^{-6}$) for AMD and 2.00 ($P = .0058$) for PCV. Among the 9 polymorphisms that were unique to the Japanese population, 2 had a minor allelic frequency of more than 0.05, and these 2 polymorphism were included as nonrisk haplotypes.

• CONCLUSIONS: The de1443ins54 polymorphism is a common variant between White and Japanese populations. It is strongly associated not only with AMD but also with PCV. (Am J Ophthalmol 2009;147:1037–1041. © 2009 by Elsevier Inc. All rights reserved.)

AJO.com Supplemental Material available at AJO.com.

Accepted for publication Dec 30, 2008.

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AGE-RELATED MACULAR DEGENERATION (AMD) IS A major cause of visual decrease in individuals in developed countries. Twin studies and segregation analyses have determined that heredity is the primary contributor to the susceptibility of patients to develop AMD.[1,2] Among the many genetic variants that contribute to AMD, complement factor H (*CFH*) and *ARMS2/HTRA1* polymorphisms have been shown frequently to be strong genetic factors for AMD, and together account for more than 50% of AMD in White persons.[3–9] These genetic associations also are seen commonly in Asian patients with exudative AMD, even though the clinical picture of AMD in the Japanese is different from that in White persons.[10,11]

Polypoidal choroidal vasculopathy (PCV) is a macular disease found in the elderly that is as prevalent as exudative AMD in the Asian population. PCV accounts for approximately 30% to 50% of the total number of eyes with senile macular diseases in elderly Asians.[12,13] Despite the lack of monozygotic or segregation studies of PCV patients, several association studies have shown that polymorphisms in the *CFH* and *ARMS2/HTRA1* genes contributed strongly to this disease.[14–16]

Recently, Fritsche and associates reported that a deletion-and-insertion polymorphism of the *ARMS2* gene, that is, NM_001099667.1:c.* 372_815de1443ins54, causes exudative AMD in White persons.[17] They showed that this polymorphism led to a decrease in the stability of the messenger ribonucleic acid of *ARMS2*, whose product is expressed in the ellipsoid of human photoreceptor cells.[17]

Differences in the distribution of polymorphisms in the human genome between populations are well established.[18] In addition to the genomic diversities, the differences in the clinical presentation of senile macular diseases between populations led us to resequence the *ARMS2* gene in Japanese individuals. The purpose of this study was to determine the characteristics of the polymorphisms in the *ARMS2* gene in Japanese individuals with AMD or PCV and in healthy controls and also to perform an association study for each polymorphism.

## METHODS

ONE HUNDRED AND EIGHTY-EIGHT UNRELATED JAPANESE individuals were studied; 56 patients had exudative AMD

0002-9394/09/$36.00
doi:10.1016/j.ajo.2008.12.036

**TABLE 1.** Polymorphisms in the *ARMS2* Gene Lesion: Distribution and Genotypes in Exudative Age-Related Macular Degeneration, Polypoidal Choroidal Vasculopathy, and Controls in the Japanese Population

| Position[a] | dbSNP ID | Location | Reference[b] | Variant[b] | Variant Freqency Cont[c] | Controls (n = 77), $Ref_{homo}$/Heter/ $Var_{homo}$ | AMD (n = 56), $Ref_{homo}$/Heter/ $Var_{homo}$ | PCV (n = 55), $Ref_{homo}$/Heter/ $Var_{homo}$ |
|---|---|---|---|---|---|---|---|---|
| 124204129 | | 5'UTR | AT | CA | 0.007 | 76/1/0 | 56/0/0 | 55/0/0 |
| 124204217 | | 5'UTR | A | G | 0.007 | 76/1/0 | 56/0/0 | 54/1/0 |
| 124204345 | rs2736911 | R38X | C | T | 0.156 | 54/22/1 | 44/12/0 | 41/12/2 |
| 124204438 | rs10490924 | A69S | G | T | 0.405 | 26/36/12 | 8/22/26 | 12/25/18 |
| 124204507 | | Intron | C | T | 0.007 | 76/1/0 | 56/0/0 | 55/0/0 |
| 124204594 | EU427528(310-311) | Intron | — | TG | 0.383 | 29/37/11 | 8/22/26 | 12/24/19 |
| 124204966 | rs36212731 | Intron | G | T | 0.383 | 29/37/11 | 8/22/26 | 12/24/19 |
| 124205188 | rs36212732 | Intron | A | G | 0.383 | 29/37/11 | 8/22/26 | 12/24/19 |
| 124205193 | | Intron | A | G | 0.000 | 77/0/0 | 56/0/0 | 54/1/0 |
| 124205201 | rs36212733 | Intron | T | C | 0.383 | 29/37/11 | 8/22/26 | 12/24/19 |
| 124205305 | rs3750848 | Intron | T | G | 0.383 | 29/37/11 | 8/22/26 | 12/24/19 |
| 124205411 | rs3750847 | Intron | C | T | 0.383 | 29/37/11 | 8/22/26 | 12/24/19 |
| 124205555 | rs3750846 | Intron | T | C | 0.383 | 29/37/11 | 8/21/26 | 11/24/19 |
| 124205584 | rs2736912 | Intron | C | T | 0.156 | 54/22/1 | 44/12/0 | 42/21/2 |
| 124206374 | rs10664316 | Intron | TA | — | 0.375 | 31/33/12 | 39/14/3 | 35/14/6 |
| 124206423 | | Intron | C | A | 0.000 | 76/0/0 | 55/1/0 | 55/0/0 |
| 124206525 | | Intron | G | A | 0.007 | 76/1/0 | 56/0/0 | 55/0/0 |
| 124206811 | EU427539(104-157) | 3'UTR | — | del443ins54 | 0.403 | 26/40/11 | 7/23/26 | 12/23/20 |
| 124207121 | | 3'UTR | C | T | | (87/5)[d] | (35/2)[d] | (44/3)[d] |
| 124207277 | | Intergenic | C | T | 0.158 | 53/22/1 | 44/12/0 | 42/10/2 |
| 124207404 | | Intergenic | T | C | 0.058 | 68/9/0 | 51/5/0 | 49/5/0 |
| 124207622 | rs2014307 | Intergenic | T | G | 0.623 | 12/34/31 | 3/14/39 | 5/14/35 |

AMD = age-related macular degeneration; Cont = controls; Heter = heterozygote; PCV = polypoidal choroidal vasculopathy; $Ref_{homo}$ = homozygote for the reference allele; SNP = single nucleotide polymorphism; $Var_{homo}$ = homozygote for the variant allele.

[a]Number of nucleotide position in NT_030059.12.

[b]Reference and variant were defined by dbSNP128. For the SNPs not registered in dbSNP128, referents were nucleotides in NT_030059.12.

[c]Allele frequency of the variation in controls.

[d]SNP is located within the deleted region of c.* 372_815delins54.

(mean age ± standard deviation [SD], 76.2 ± 9.12 years; ratio of men to women, 36:20) and 55 patients had PCV (mean age ± SD, 72.9 ± 7.28 years; ratio of men to women, 45:10). For controls, 77 individuals without age-related maculopathy (ARM) were studied (mean age, 73.4 ± 8.96 years; ratio of men to women, 35:42). The definitions of exudative AMD and ARM were those of the International Classification System for ARM, but grading was not performed.[19] The diagnosis of PCV was based on indocyanine green angiography (ICGA) results, which showed a branching vascular network that terminated in aneurysmal enlargements, that is, polypoidal lesions. All cases with exudative AMD and PCV received a general ophthalmologic examination including fluorescein angiography and ICGA with HRA2 (Heidelberg Engineering, Heidelberg, Germany). Eyes with other macular abnormalities, for example, pathologic myopia, idiopathic choroidal neovascularization (CNV), presumed ocular histoplasmosis, angioid streaks, and other secondary CNV, were excluded.

Genomic deoxyribonucleic acid samples were prepared with QuickGene-610L (Fujifilm; Minato, Tokyo, Japan). Polymerase chain reaction amplification of chromosome 10:124,203,961-124,207,675 (NC_000010.9, NCBI Build 38), which covers the entire *ARMS2* gene (GeneID, 387715, NM_001099667), was performed with LATaq with GC buffer (TaKaRa Bio, Ohtsu, Japan). The products were sequenced directly according to protocols accompanying the ABI BigDye terminator kit version 3.1 using an ABI PRISM 3730 DNA Analyzer (Applied Biosystems, Foster City, California, USA). Primers for the polymerase chain reaction amplification and sequence reactions are shown in the Supplemental Table available at AJO.com. The alignment of the sequences, detection of polymorphisms, and genotyping were performed with Genalys version 3.4.8a.[20]

Deviations in the genotype distributions from the Hardy–Weinberg equilibrium (HWE) were assessed with the HWE exact test. Allelic distributions were compared between patients and controls by using the Chi-square test for

**TABLE 2.** Haplotype Analysis of *ARMS2* Gene Lesion in Exudative Age-Related Macular Degeneration, Polypoidal Choroidal Vasculopathy, and Controls in the Japanese Population

| | Estimated Frequency | | | Association Results | | | |
|---|---|---|---|---|---|---|---|
| | | | | AMD vs Control | | PCV vs Control | |
| Haplotypes[a] (>1%) | AMD (n = 56) | PCV (n = 55) | Controls (n = 77) | Odds Ratio (95% CI)[b] | *P* value | Odds Ratio (95% CI)[b] | *P* value |
| CT2TGCGTCCT2CTG | 0.661 | 0.555 | 0.383 | 3.14 (1.89 to 5.21) | $7.8 \times 10^{-6}$ | 2.00 (1.21 to 3.29) | .0058 |
| CG1GATTCTCC1CTT | 0.179 | 0.236 | 0.370 | 0.37 (0.21 to 0.66) | $7.0 \times 10^{-4}$ | 0.53 (0.30 to 0.91) | .021 |
| TG1GATTCTTT1TTG | 0.107 | 0.127 | 0.156 | 0.61 (0.30 to 1.26) | .25 | 0.74 (0.37 to 1.47) | .51 |
| CG1GATTCTCT1CCG | 0.045 | 0.045 | 0.058 | 0.75 (0.24 to 2.31) | .62 | 0.77 (0.24 to 2.36) | .64 |

AMD = age-related macular degeneration; CI = confidence interval; PCV = polypoidal choroidal vasculopathy.

[a]Polymorphisms are in the following order: rs2736911, rs10490924, EU427528(310-311), rs36212731, rs36212732, rs36212733, rs3750848, rs3750847, rs3750846, rs2736912, rs10664316, EU427539(104-157), J8, J9, rs2014307. In 2 polymorphisms that are not a single nucleotide polymorphism, reference sequence appears as 1, and the variant appears as 2.

[b]Odds ratios are given for the specified haplotype compared with all other pooled haplotypes.

independence. Linkage disequilibrium (LD) and haplotypes were assessed with Haploview, version 4.1.

## RESULTS

THE DISTRIBUTIONS OF THE POLYMORPHISMS AND GENOtypes of the *ARMS2* gene for the 188 participants are presented in Table 1. In the 77 Japanese normal controls, 20 polymorphisms (16 single nucleotide polymorphisms [SNPs] and 4 insertions and deletions) were identified. Of these, 7 polymorphisms (6 SNPs, 1 insertion and deletion) were not reported by Fritsche and associates, and all except SNP(C/T) at the position of 124207277 and SNP(T/C) at the position of 124207404 were seen as singletons.[17] However, 8 polymorphisms, including rs10490923 (R3H) in this genomic region, that were reported in White persons were not detected in the Japanese individuals.[17] All identified polymorphisms in the controls were in HWE ($P > .624$). An extension of LD throughout the *ARMS2* gene was detected ($r^2 > 0.98$).

In the 56 exudative AMD patients, 17 polymorphisms were identified. Among them, 1 SNP was identified as a unique polymorphism that was not seen in controls, but it was identified in only 1 patient. All identified polymorphisms were in HWE ($P > .350$). Seventy-five del443ins54 polymorphisms were resequenced, and the sequences were identical to those reported in White persons.[17]

Haplotypes were inferred for 15 common polymorphisms, and case-control association studies were conducted for these haplotypes (Table 2). The haplotype with the highest prevalence in the controls had an odds ratio of 3.14 (95% confident interval, 1.89 to 5.21; $P = 7.8 \times 10-^{6}$) in Japanese with AMD. The coding for SNP rs273911, which would result in a premature stop (R38X) in the ARMS2 protein, was classified as a nonrisk haplotype, as in that reported in White persons.[17] Among the 9 polymorphisms that were unique to the Japanese population, 2 had a minor allelic frequency of more than 0.05, and these polymorphism were included as a nonrisk haplotype.

For the 55 PCV patients, 18 polymorphisms were identified. Among them, 1 SNP that was not seen in controls was identified in 1 patient as a unique polymorphism. All identified polymorphisms were in HWE ($P > .053$). Sixty-three alleles of the del443ins54 polymorphism were resequenced, and again there was no difference from that found in White persons.[17] Haplotypes were inferred as in the AMD cases, and the odds ratio by risk haplotype in PCV did not show any significant differences compared with that of AMD (Table 2). The coding SNP rs273911 (R38X) was included as a nonrisk haplotype as in the AMD cases.

## DISCUSSION

WE SYSTEMATICALLY EXAMINED THE *ARMS2* GENE FOR polymorphisms in Japanese cohorts and found that the del443ins54 is a variant in Japanese persons, as it was in White persons. In the Japanese, the haplotype that contained the del443ins54 polymorphism was strongly associated not only with exudative AMD but also with PCV.

Chromosome 10q26 has been reported to carry an important AMD susceptibility locus in both the White and Asian populations. Several studies have used refined LD mapping and case-control association studies to probe the most susceptible alleles, *ARMS2* rs10490924 and *HTRA1* rs11200638, for AMD.[5,7,10,21,22] There is a strong LD across the *ARMS2/HTRA1* region, making genetic association studies alone not sufficient to distinguish between the 2 candidates.

Recent functional studies have revealed the role played by this region in pathologic features of AMD. Thus, Kanda and associates showed that the product of the *ARMS2* gene was found in mitochondria,[22] and Fritshe and associates

showed that the ARMS2 protein is located in the ellipsoid, a mitochondria-concentrated part of human photoreceptor cells. They also showed that the level of expression of this protein is associated with de1443ins54 variant, which directly affects the amount of transcript by removing the polyadenylation signal and inserting a 2 AUUUA signal known to mediate rapid messenger ribonucleic acid turnover.[17] In addition, the hypothesis that *ARMS2* contributes to the pathogenesis of AMD is supported by clinical observations. For example, Baer and associates evaluated the recurrence pattern of CNV after macular translocation surgery for neovascular AMD and reported that recurrences of CNV were most common at the edge closest to the new fovea.[23] They proposed that the development of CNV occurs by a signaling mechanism at the fovea, where many photoreceptors are concentrated.

Considering these reports, we focused on identifying polymorphisms in the *ARMS2* gene, and so did not study the *HTRA1* gene region. Far more studies will be needed to examine gene polymorphisms in the *HTRA1* gene in the Japanese population.

In addition to exudative AMD, PCV is another important macular disease in elderly Asians. PCV was described first by Yannuzzi and associates (Yannuzzi LA. Idiopathic polypoidal choroidal vasculopathy. Presented at the Macula Society Meeting, February 5, 1982, Miami, Florida.), who named it *idiopathic polypoidal choroidal vasculopathy*. After a decade of many studies, it has been well established that PCV is a distinct macular disease and ICGA is mandatory to make a diagnosis.[24,25] The differentiation of PCV from exudative AMD is very important because of differences not only in the natural history but also in the response to treatments, including photodynamic therapy.[26,27] Although there is no consensus on what makes these 2 macular conditions different, it is noteworthy that PCV is seen more frequently in Asians, African Americans, and Hispanics, suggesting some genetic influence.[25,27] Kondo and associates reported that a polymorphism of the elastin gene is associated with PCV, and they reported that different pathogenic processes are involved in the phenotypic expression of neovascular AMD and PCV.[28]

However, our findings of the association of the *ARMS2* region with PCV patients was very similar to that of exudative AMD patients. The association on 10q26 with PCV has been shown in earlier studies.[14–16] Interestingly, the discrimination of the 2 conditions is sometimes very difficult in the clinical setting, even if the highest quality ICGA images are obtained. Although we carefully selected indisputable cases for this study, Maruko and associates reported that 5.5% of Japanese exudative AMD patients have a combined pattern of typical exudative AMD and PCV.[13] Because the number of patients is limited, it difficult to interpret the results in detail, and it may well be that patients with AMD and PCV have a common genetic background. We suggest that the pathway to CNV may be the same for both AMD and PCV, and PCV may be 1 subtype of occult CNV in the FA pattern.[24,29,30] Then, another genetic background may contribute to the maturation of the vessels to present different clinical pictures.

The gender ratio was different between the cases and controls, and this is a limitation of this study. However, there have been several studies targeting 10q26 for exudative AMD or PCV in Asian populations, and all these studies reported that differences between genders existed. In addition, all of these studies reported that the association of 10q26 is effective even after gender correction.[10,15,31,32] So, future studies of Asian populations are needed to determine the genetic contribution of 10q26 to AMD and PCV with the gender differences eliminated.

A difference in the distribution of the polymorphisms that cause the more common ocular diseases have been found in different ethnic populations.[11,33–35] However, our results on the *ARMS2* gene showed that the conclusions drawn from a functional study for *ARMS2* are similar for both White and Asian populations.

In summary, our data confirmed that the incidence of 1 of the *ARMS2* polymorphisms is similar in white and Japanese populations. In addition, this polymorphism is strongly associated not only with AMD, but also with PCV. Determining the relationship of the genetic background and the diversity of the clinical characteristics between the ethnic groups will require further studies.

THIS STUDY WAS SUPPORTED IN PART BY THE MINISTRY OF EDUCATION, CULTURE, SPORTS, SCIENCE AND TECHNOLOGY of Japan, Chiyoda, Tokyo, Japan and by Japan National Society for the Prevention of Blindness, Shinjuku, Tokyo, Japan. The authors indicate no financial conflict of interest. Involved in design of study (N.G., R.Y., N.Y.); conduct of laboratory study (N.G., N.H., H.H.); collection of samples (N.G., H.N., H.H., A.O., A.T., K.Y., H.T., M.S., K.S., T.I.); management (F.M., N.Y.), analysis (N.G.), and interpretation of data (N.G., N.H., H.H., N.Y.); preparation of the manuscript (N.G., N.Y.); and approval of the manuscript (N.Y.). This study was performed in accordance the Declaration of Helsinki and was approved by the Institutional Review Board (IRB) of Kyoto University Graduate School of Medicine and the IRB of the Fukushima Medical University. All personal information associated with the blood samples was encrypted.

The authors thank Drs Yoshiki Ueda, Nagahama City Hospital, Nagahama, Japan and Shoji Kuriyama, Otsu Red Cross Hospital, Otsu, Japan, for their assistance in recruiting participants.

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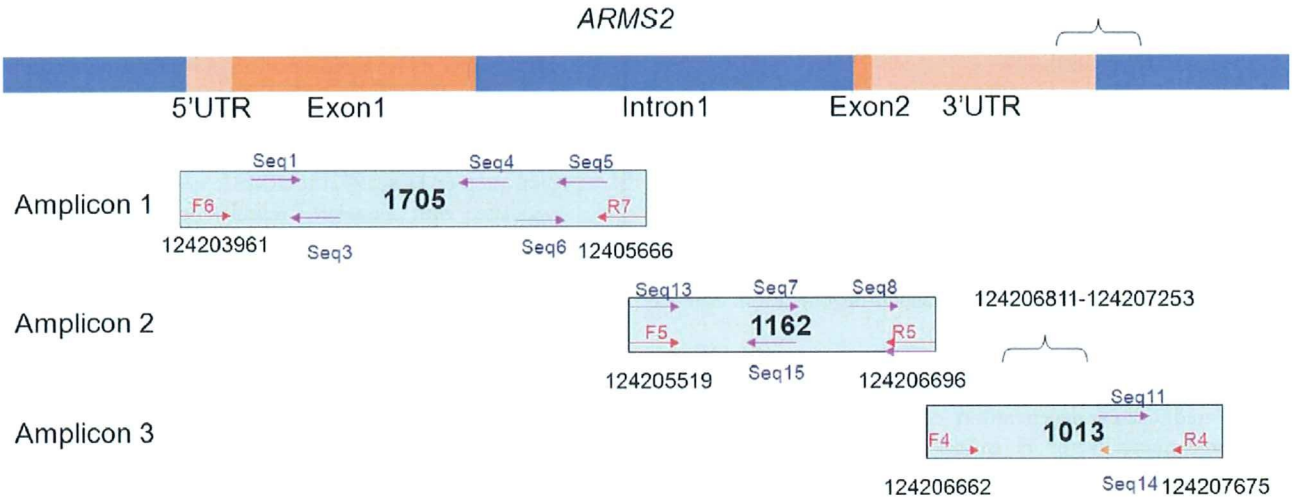
**SUPPLEMENTAL TABLE.** Sequence Primers for *ARMS2* Gene Lesion

124,203,961-124,207,675 (NC_000010.9, NCBI Build 38)




Primers for PCR Fragment

| | Primer Name | Forward | Primer Name | Reverse |
|---|---|---|---|---|
| Amplicon1 | ARMS2_F6 | TTCAAATCCCTGGGTCTCTG | ARMS2_R7 | CTGCTGCTGCTCAGTTTCCT |
| Amplicon2 | ARMS2_F5 | TGGAAGGAGCCAATTAATGC | ARMS2_R5 | GATTTCACCAGCTGCACAGA |
| Amplicon3 | ARMS2_F4 | TGGATGCATCTTCTGCTCTG | ARMS2_R4 | TTTTGAACAGGGCCAAGTCT |

Sequencing Primers

| | Primer Name | | Direction | Location |
|---|---|---|---|---|
| Amplicon1 | ARMS2_Seq1 | GACCTCTGTTGCCTCCTCTG | Forward | 124,204,023-124,204,042 |
| Amplicon1 | ARMS2_Seq3 | GTCACTGGCTCCTTCTCCAC | Reverse | 124,204,373-124,204,392 |
| Amplicon1 | ARMS2_Seq4 | GCCACAGTTCCGTAAGCATT | Reverse | 124,205,154-124,205,173 |
| Amplicon1 | ARMS2_Seq5 | TGCATTAATTGGCTCCTTCC | Reverse | 124,205,520-124,205,539 |
| Amplicon1 | ARMS2_Seq6 | GCCTAAATGGCAGCATGTTC | Forward | 124,205,317-124,205,336 |
| Amplicon2 | ARMS2_Seq13 | CTGCTGGTCTCATGCAACTG | Forward | 124,205,548-124,205,567 |
| Amplicon2 | ARMS2_Seq7 | GGCAAATCACTTGCAGTCAG | Forward | 124,205,933-124,205,952 |
| Amplicon2 | ARMS2_Seq8 | ATCATCCACACTGCAGCAAG | Forward | 124,206,416-124,206,435 |
| Amplicon2 | ARMS2_Seq15 | GATAGAGTCTCACTCTGTTG | Reverse | 124,206,138-124,206,157 |
| Amplicon2 | ARMS2_R5 | GATTTCACCAGCTGCACAGA | Reverse | 124,206,677-124,206,696 |
| Amplicon3 | ARMS2_F4 | TGGATGCATCTTCTGCTCTG | Forward | 124,206,661-124,206,680 |
| Amplicon3 | ARMS2_R4 | TTTTGAACAGGGCCAAGTCT | Reverse | 124,207,655-124,207,674 |
| Amplicon3 | ARMS2_Seq11 | TGGGAGAATGGGTGAGTAGG | Forward | 124,207,248-124,207,267 |
| Amplicon3 | ARMS2_Seq14 | TTGGCTCCTCCTATAAGCT | Reverse | 124,207,411-124,207,429 |

## Biosketch

Norimoto Gotoh, MD, graduated from Shinshu University School of Medicine, and obtained his MD in 2000. He completed his residency program at Shinshu University Hospital, Matsumoto, Japan under Dr Nagahisa Yoshimura. In 2004, he investigated ocular genomics at the Department of Ophthalmology and Center for Genomic Medicine at Kyoto University Graduate School of Medicine. Dr Gotoh's main interest is the genetic contribution to ocular diseases. In 2009, he will join the laboratory of Dr Anand Swaroop at the National Eye Institute.

*Journal of Viral Hepatitis*, 2009, **16**, 388–396 doi:10.1111/j.1365-2893.2009.01095.x

# Association of genetic polymorphisms with interferon-induced haematologic adverse effects in chronic hepatitis C patients

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*Received August 2008; accepted for publication November 2008*

SUMMARY. Interferon (IFN)-based combination therapy with ribavirin has become the gold standard for the treatment of chronic hepatitis C virus infection. Haematologic toxicities, such as neutropenia, thrombocytopenia, and anaemia, however, frequently cause poor treatment tolerance, resulting in poor therapeutic efficacy. The aim of this study was to identify host genetic polymorphisms associated with the efficacy or haematologic toxicity of IFN-based combination therapy in chronic hepatitis C patients. We performed comprehensive single nucleotide polymorphism detection in all exonic regions of the 12 genes involved in the IFN signalling pathway in 32 healthy Japanese volunteers. Of 167 identified polymorphisms, 35 were genotyped and tested for an association with the efficacy or toxicity of IFN plus ribavirin therapy in 240 chronic hepatitis C patients. Multiple logistic regression analysis revealed that low viral load, viral genotypes 2 and 3, and a lower degree of liver fibrosis, but none of the genetic polymorphisms, were significantly associated with a sustained virologic response. In contrast to efficacy, multiple linear regression analyses demonstrated that two polymorphisms (*IFNAR1* 10848-A/G and *STAT2* 4757-G/T) were significantly associated with IFN-induced neutropenia ($P = 0.013$ and $P = 0.011$, respectively). Thrombocytopenia was associated with the *IRF7* 789-G/A ($P = 0.031$). In conclusion, genetic polymorphisms in IFN signalling pathway-related genes were associated with IFN-induced neutropenia and thrombocytopenia in chronic hepatitis C patients. In contrast to toxicity, the efficacy of IFN-based therapy was largely dependent on viral factors and degree of liver fibrosis.

*Keywords*: haematologic adverse effect, hepatitis C, interferon, single nucleotide polymorphism, sustained virologic response.

## INTRODUCTION

Hepatitis C virus (HCV) infects an estimated 170 million people worldwide [1] and is a leading cause of chronic hepatitis, liver cirrhosis, and primary hepatocellular carcinoma [2]. Currently, combination therapy with ribavirin (RBV) and either conventional interferon (IFN)-α or pegylated-IFN-α (peg-IFN-α) is the gold standard of treatment for chronic HCV infection [3,4], but the overall rate of a sustained virologic response (SVR) with these therapies ranges from only 54% to 63% [5–7]. The limited therapeutic efficacy might be due to the poor virologic response in some patients or to adverse effects of the IFN-based therapy, leading to low treatment tolerance [5,6].

Predictive factors associated with a virologic response to IFN-based therapy include viral and host factors. Several studies have recently reported a possible association between the efficacy of IFN-based therapy and polymorphisms in genes encoding cytokines, chemokines, or their receptors [8–14]. The reported single nucleotide polymorphisms (SNPs) associated with a virologic response to IFN-based therapy include the *IFNAR1* [8], *IL-10* [9,10], *TNF-α* [11], *IFN-γ* [12], *CCR5* [13], *osteopontin* [14] and *TLR7* [15] genes. These data, however, are controversial and inconclusive, because most of the previous studies analysed a selected single target gene. Indeed, such limited evaluation of only one or two SNPs might not be sufficient in determining association of genetic polymorphisms with a virologic response to IFN-based therapy. Moreover, few studies have involved patients treated with combination therapy using peg-IFN-α and RBV [16,17].

Abbreviations: ALT, alanine aminotransferase; CI, confidence interval; HCV, hepatitis C virus; IFN, interferon; OR, odds ratio; PCR, polymerase chain reaction; RBV, ribavirin; SNP, single nucleotide polymorphism; SVR, sustained virologic response.

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Among the side effects of IFN plus RBV combination therapy, haematologic toxicities are frequently observed and sometimes treatment must be discontinued or the drug dose reduced, resulting in reduced efficacy of the combination therapy [5,6,18]. However, the mechanisms and predictive factors in the occurrence of these adverse effects, especially the critical decrease in blood cell count, are not clear at present.

Many studies have clarified the molecular pathway of action of IFN in detail [4,19,20]. Binding of IFN-α to its receptor induces IFNAR1 and IFNAR2 dimerization, followed by the activation of IFNAR-associated tyrosine kinases (JAK1 and TYK2). These tyrosine kinases phosphorylate STAT1 and STAT2 monomers, leading to the induction of multiple IFN-stimulated genes. Moreover, type I IFNs induce IRF7 and IRF3, which are responsible for type 1 IFN induction mediated by the virus or Toll like receptors [21]. On the other hand, the mechanisms of IFN induction in response to viral infection were recently determined [22,23]. In HCV-infected cells, the cytoplasmic RNA helicase, RIG-I, recognizes the viral dsRNA and interacts with IPS-1, leading to activation of the transcription factors, IRF3 and NF-kB, which in turn transcribe type I IFN genes. In contrast, IRF2 negatively regulates the IFN signalling pathway and recent studies suggest that IRF2 modulates the differentiation of haematopoietic cells [24–26]. Despite the unveiling of the molecular pathway of IFN signalling, it remains unclear why IFN-based therapy induces divergent efficacy or adverse haematologic toxicities in different patients.

In the present study, therefore, in order to determine the genetic factors associated with not only the efficacy but also haematologic toxicity of IFN-based therapy, we focused on the genes involved in the IFN signalling pathway, and performed a large-scale and comprehensive analysis of the genetic polymorphisms in 12 genes among chronic hepatitis C patients receiving IFN plus RBV therapy. To identify the predictors of efficacy or haematologic toxicity of IFN-based therapy, we carried out multivariate analyses using various clinicopathological factors and genetic polymorphisms.

## MATERIALS AND METHODS

### *Patients*

DNA for SNP screening was extracted from blood samples of 32 healthy Japanese volunteers under the auspices of the Pharma SNP Consortium (Tokyo, Japan). The participants comprised 240 Japanese adult chronic hepatitis C patients receiving conventional IFN-α 2b ($n$ = 157) or peg-IFN-α 2b ($n$ = 83) plus RBV combination therapy (Schering-Plough, Kenilworth, NJ, USA) at Kyoto University and affiliated hospitals from February 2002 to August 2007. In Japan, peg-IFN-α 2b plus RBV combination therapy was approved in October 2004. Thus, the patients who participated before and after October 2004 received conventional IFN-α 2b and peg-IFN-α 2b, respectively. Indications for IFN-based therapy included high serum values of alanine aminotransferase (ALT) and positivity for serum anti-HCV and HCV RNA. Histological examination of liver biopsy specimens was avail able for 165 (68.8%) of the 240 enrolled patients. Liver histology was assessed by an experienced hepatopathologist using the METAVIR score [27]; the fibrosis stage was defined as: F0 (no fibrosis), F1 (mild fibrosis), F2 (moderate fibrosis), F3 (severe fibrosis) and F4 (cirrhosis). The ethics committee at Kyoto University approved the studies, and informed consent for participation in the study was obtained from all patients.

### *IFN-α 2b or peg-IFN-α 2b plus RBV combination therapy*

Patients receiving conventional IFN-α plus RBV therapy were treated with 6 million units of recombinant IFN-α 2b daily for 2 weeks and with 6 million units three times a week for the following assigned treatment period, in combination with daily oral RBV. The RBV dose was 600 mg/day in patients weighing less than 60 kg, and 800 mg/day in those weighing 60 kg or more. Patients receiving peg-IFN-α 2b plus RBV therapy were treated with peg-IFN-α 2b once per week, combined with daily oral RBV for the assigned period. The peg-IFN-α 2b dose was 1.5 μg/kg per week. Patients with genotype 1 received 48 weeks of combination therapy and patients with genotypes 2 and 3 received 24 weeks of combination therapy.

The dosage of IFN-α 2b or peg-IFN-α 2b was reduced by half if platelet counts dropped to <80 000/μL, if leucocyte counts dropped to <1500/μL, or if neutrophil counts dropped to <750/μL during therapy. IFN-α 2b or peg-IFN-α 2b was discontinued if platelet counts dropped to <50 000/μL, if leucocyte counts dropped to <1000/μL, or if neutrophil counts dropped to <500/μL during therapy. The RBV dosage was reduced to 400 mg/day or 600 mg/day if haemoglobin levels were less than 10 g/dL. RBV was discontinued if haemoglobin levels were less than 8.5 g/dL.

Sustained virologic response was defined as no detectable HCV RNA by qualitative assay for at least 24 weeks after cessation of therapy. Non-SVR was defined as no response or relapse after the cessation of therapy.

### *SNP screening of the IFN signalling pathway-related genes*

We selected the following IFN signalling pathway-related genes, including seven genes involved in the intracellular IFN-mediated signalling pathway from the binding of IFN to its receptor to initiation of the transcription of various target genes [20]; four genes involved in the RIG-I signalling pathway, which triggers the IFN-induction pathway after viral infection [22,23], and one gene that negatively regulates the IFN signalling pathway [24] [IFNAR1 (NT_011512.10, NM_000629.2), IFNAR2 (NT_011512.10, NM_207585.1), JAK1 (NT_032977.7, NM_002227.1), TYK2 (NT_011295.10, NM_003331.3), STAT1 (NT_005403.15, NM_007315.2), STAT2 (NT_029419.10, NM_005419.2), IRF9 (NT_026437.11, NM_006084.3), RIG-I (NT_

008413.16, NM_014314.2), IPS-1 (NT_011387.8, NM_020746.1), IRF3 (NT_011109.15, NM_001571.2), IRF7 (NT_035113.6, NM_004031.1), and IRF2 (NT_0022792.17, NM_002199.3)]. Genomic DNA was extracted from blood samples of 32 healthy Japanese volunteers using a DNA extraction kit (Genomix Kit; TALENT, Trieste, Italy), and the 179 exons, including the 5′- and 3′-untranslated regions and adjacent intronic regions of the 12 candidate genes, were amplified. The resultant polymerase chain reaction (PCR) products were used as templates for direct sequencing on an ABI 3730 automated sequencer (Applied Biosystems, Foster City, CA, USA). Segregating sites were identified and genotypes were confirmed directly from electrophorograms using Genalys (http://www.software.cng.fr/docs/genalys.html) [28].

### *SNP genotyping*

Among the SNPs identified by the screening, we selected tag SNP markers that covered all of the common (>5% frequency) haplotypes using the minimal haplotype tagging method, one of the best methods to identify the smallest tagging set for an arbitrary region of the genome [29]. These tag SNPs allowed us to genotype the smallest possible number of SNPs for each gene while resolving all common haplotypes. We also included SNPs that existed in coding sequences or 5′ flanking regions with frequencies higher than 5%. These SNPs were genotyped using the ABI Taqman allelic discrimination method and an ABI 7900HT sequence detection system (Applied Biosystems). Primers and probes were designed by the manufacturer with SNP browser Software (Applied Biosystems), as shown in Tables S1 and S2. Amplification reactions were performed in a 3 μL volume, with 5 ng DNA, 1.5 μL universal PCR master-mix, and 0.0375 μL assay mix with the specific primers and probes. Seven SNPs that could not be detected using the Taqman assay were determined by direct sequencing of PCR products amplified with primers specific for each SNP (Table S3).

### *Statistical analysis*

Genotype distributions were tested for Hardy–Weinberg equilibrium using exact tests. To identify predictors of SVR, we used univariate analysis of pre-treatment factors to compare all SVR and non-SVR patients who had completed the treatment. The following pre-treatment factors were considered: SNPs, sex (male *vs* female), age (in years), weight (in kilograms), serum ALT, IFN history (naive *vs* relapse *vs* nonresponse), HCV genotype (1 *vs* 2 and 3), HCV viral load (<100 *vs* 100 to <500 *vs* 500 to <850 *vs* ≥850 kIU/mL), and fibrosis stage (F0 *vs* F1 *vs* F3 *vs* F4). Allele and genotype frequencies were evaluated for their association with SVR using Fisher's exact tests. Sex, IFN history, and HCV genotype were evaluated using the chi-square test. Age, weight, and serum ALT were evaluated using the Mann–Whitney *U*-test. Fibrosis stage and viral load were evaluated using a trend chi-square test. We considered two-tailed *P*-values <0.05 to be statistically significant and calculated odds ratios (ORs) and 95% confidence intervals. Multiple logistic regression analysis was performed using STATISTICA (StatSoft, Tulsa, OK, USA) to evaluate the association between SVR and significant factors from the univariate analyses.

To identify predictors of cytopenia, we examined the association between decreased leucocyte, neutrophil, and platelet counts and haemoglobin levels, and the following patient characteristics and clinical features using linear regression analysis with STATISTICA: sex, age, weight, fibrosis stage and SNPs. Multiple linear regression analysis was performed to evaluate the association between the decreased peripheral blood cell numbers and significant factors from the univariate analyses.

## RESULTS

### *Genetic variations and polymorphisms in IFN signalling pathway-related genes*

By screening 32 healthy volunteers, we identified 167 genetic polymorphisms (153 SNPs and 14 insertions/deletions) in the 12 IFN signalling pathway-related genes (Table 1, Table S4). All identified polymorphisms were in Hardy–Weinberg equilibrium. Of these 167 polymorphisms, 60 (49 SNPs and 11 insertions/deletions) were novel and were not registered in Build 125 of the SNP database (http://www.ncbi.nlm.nih.gov) (Table 2). Among the 167 SNPs identified, 30 (16 nonsynonymous and 14 synonymous) were located in exons and we confirmed that 14 of the 30 SNPs identified in the exons were novel. Furthermore, we identified 10 novel nonsynonymous variants in the seven genes. Sixty-two SNPs were relatively uncommon (minor allele frequency <0.05) and were thus excluded from further analysis. Finally, 27 selected tag SNPs and eight additional SNPs that existed in coding sequences or 5′ flanking regions were subjected to further genotyping analyses in chronic hepatitis C patients (Table 2).

### *Variables associated with virologic response to IFN-based therapy*

The relationship between baseline characteristics and virologic response to the IFN plus RBV combination therapy in chronic hepatitis C patients is summarized in Table 3. Combination therapy was discontinued in 37 patients during the assigned treatment period. These 37 patients were excluded from analysis of the virologic response. SVR was achieved in 98 of 203 (48.3%) patients, and 105 patients (51.7%) had a relapse of HCV infection after the end of therapy or showed no response to IFN-based therapy.

To determine the predictive factors for IFN-based therapy efficacy, we examined the correlation between virologic response, and clinical and viral factors. Of 56 patients with

**Table 1** Classification of polymorphisms identified in the IFN-signalling related genes

| Gene | Number of base pairs screened | SNP | Ins/del | Total polymorphisms | Distribution by gene structure | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | 5′FL | 5′UTR | CDS (sSNP, nsSNP) | Intron | 3′UTR | 3′FL |
| IFNAR1 | 7522 | 8 | 2 | 10 | 2 | 1 | 0 (0, 0) | 5 | 2 | 0 |
| IFNAR2 | 4849 | 6 | 0 | 6 | 0 | 0 | 1 (0, 1) | 4 | 1 | 0 |
| JAK1 | 11312 | 18 | 2 | 20 | 0 | 0 | 2 (2, 0) | 18 | 0 | 0 |
| TYK2 | 8270 | 19 | 0 | 19 | 0 | 0 | 8 (3, 5) | 10 | 1 | 0 |
| STAT1 | 10647 | 20 | 3 | 23 | 0 | 0 | 3 (3, 0) | 18 | 1 | 1 |
| STAT2 | 8646 | 13 | 1 | 14 | 0 | 0 | 1 (0, 1) | 11 | 2 | 0 |
| IRF9 | 3171 | 3 | 0 | 3 | 0 | 0 | 0 (0, 0) | 2 | 1 | 0 |
| RIG-I | 8819 | 26 | 1 | 27 | 0 | 1 | 4 (1, 3) | 19 | 3 | 0 |
| IPS-1 | 5105 | 11 | 1 | 12 | 0 | 0 | 3 (1, 2) | 2 | 7 | 0 |
| IRF3 | 3968 | 8 | 2 | 10 | 0 | 3 | 2 (1, 1) | 4 | 0 | 1 |
| IRF7 | 2589 | 8 | 1 | 9 | 1 | 0 | 4 (2, 2) | 4 | 0 | 0 |
| IRF2 | 5668 | 13 | 1 | 14 | 0 | 0 | 2 (1, 1) | 10 | 1 | 1 |
| Total | 74898 | 153 | 14 | 167 | 3 | 5 | 30 (14, 16) | 107 | 19 | 3 |

SNP, single nucleotide polymorphism; ins, insertion; del, deletion; UTR, untranslated region; FL, flanking region; CDS, coding region; sSNP, synonymous SNP; nsSNP, nonsynonymous SNP.

**Table 2** One hundred and sixty-seven polymorphisms in the IFN-signalling related genes

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| IFNAR1 (10 polymorphisms) | **−347** | −6* | 51 | 10595 | **10848** | 10927 | 24135 | 24469* |
| | 33483* | **33741** | | | | | | |
| IFNAR2 (6 polymorphisms) | 14693 | 14983 | 22299* | **22687** | 33267* | 34057* | | |
| JAK1 (20 polymorphisms) | 91 | 365 | 12755 | 13212 | 13242* | 21305 | 30599 | 30856* |
| | 34934* | 34999 | 35312 | 38993 | 39038 | **40725** | 40870 | 40871 |
| | **41498** | 42571* | 46465 | 51217* | | | | |
| TYK2 (19 polymorphisms) | 2243* | 12345* | 12529 | 14003* | **14006** | 14145* | 15192* | 15452 |
| | **15560** | 18074 | 18164* | 18279 | 21293* | 26247* | 26378* | **26525** |
| | 26561* | 26854 | 29721* | | | | | |
| STAT1 (23 polymorphisms) | 283 | 821* | **4270** | 5384 | 6630* | 6751 | 16036 | 16151 |
| | 16539 | 23416 | 24514 | **27161*** | 27452 | 28838 | 30625 | 34532* |
| | 35386* | 35574* | 37058 | 37178 | 39478 | 44152* | **45397** | |
| STAT2 (14 polymorphisms) | 88 | 3706 | 3765* | **4757*** | **4901*** | 9465 | 9488 | 9634 |
| | 9819 | 10543 | 11441* | 16088* | 18063 | 18306* | | |
| IRF9 (3 polymorphisms) | 621* | **1129*** | 4265* | | | | | |
| RIG-I (27 polymorphisms) | **90** | 177 | 354 | 391* | 408 | **33794*** | 33971 | **35083** |
| | 35263 | 37764 | 38008* | 38086 | 41043 | 46072 | 49075* | 53235 |
| | 58363* | 58590* | 58615* | 59861* | 60046* | 60133* | 60139 | **66873** |
| | **69596** | 69667 | 70306 | | | | | |
| IPS1 (12 polymorphisms) | 10717 | 10748 | **10952*** | **15495** | **15538** | 18908 | **19354** | 19653 |
| | 19836* | **20479*** | 20921* | 20927 | | | | |
| IRF3 (10 polymorphisms) | **95** | 175 | 188 | 244 | 418* | 1389 | 2320 | **2652** |
| | 6206 | 6304* | | | | | | |
| IRF7 (9 polymorphisms) | −198 | 390* | **457** | **789** | 1335 | 1598* | 2488 | 2686 |
| | **2829*** | | | | | | | |
| IRF2 (14 polymorphisms) | 45305 | 45371* | 45420 | 55441* | 56210 | **66675** | 75602* | **83546** |
| | 83649* | 83700* | 83749* | **85509** | 86327 | 87066 | | |

Gene number is expressed as the nucleotide position from the first nucleotide of the transcriptional start codon. Polymorphisms in boldface are selected as tag SNP markers.
*Newly discovered polymorphisms.