**Table 3** Demographic, virological and clinical features of patients with chronic hepatitis C treated by IFN plus ribavirin combination therapy

| Variable | SVR ($n$ = 98)* | Non-SVR ($n$ = 105)* | $P$-value |
|---|---|---|---|
| Sex | | | |
| Male | 64 | 50 | **0.011** |
| Female | 34 | 55 | |
| Age (years)† | 56.0 (24–72) | 58.5 (27–74) | **0.023** |
| Weight (kg)† | 61.8 (43–91) | 61.9 (41–94) | 0.821 |
| Pre-treatment ALT (IU/L)† | 69 (17–285) | 57 (16–304) | 0.170 |
| Interferon history‡ | | | |
| Naive | 58 (51.8%) | 54 (48.2%) | 0.311 |
| Relapse | 30 (47.6%) | 33 (52.4%) | |
| Nonresponse | 10 (35.7%) | 18 (64.3%) | |
| HCV genotype | | | |
| 1 | 50 (34.0%) | 97 (66.0%) | **0.00000002** |
| 2, 3 | 48 (85.7%) | 8 (14.3%) | |
| HCV RNA titre (kIU/mL) | | | |
| <100 | 15 (93.7%) | 1 (6.3%) | **0.004** |
| 100–500 | 35 (49.3%) | 36 (50.7%) | |
| 500–850 | 22 (44.9%) | 27 (55.1%) | |
| 850≤ | 26 (38.8%) | 41 (61.2%) | |
| Fibrosis score | | | |
| 0 | 5 (62.5%) | 3 (37.5%) | **0.0005** |
| 1 | 38 (59.4%) | 26 (40.6%) | |
| 2 | 19 (45.2%) | 23 (54.8%) | |
| 3 | 5 (20.0%) | 20 (80.0%) | |
| 4 | 2 (28.6%) | 5 (71.6%) | |

SVR, sustained virologic response. $P$-values in boldface are significant.
*SVR and non-SVR were evaluated in patients who had completed therapy for 24 or 48 weeks.
†Values are median (range).
‡One hundred six patients had received previous treatment with IFN-α monotherapy for 24 weeks, but failed to respond or relapsed.

HCV genotypes 2 and 3, 48 (85.7%) had SVR, whereas 50 of 147 (34.0%) patients with HCV genotype 1 had SVR, indicating that HCV genotype 1 was significantly associated with non-SVR ($P$ = 0.00000002). In addition, a lower viral load before treatment ($P$ = 0.004), male sex ($P$ = 0.011), young age ($P$ = 0.023), and lower degree of liver fibrosis ($P$ = 0.0005) were significantly associated with SVR.

SNP genotyping analyses revealed that the frequencies of all 35 polymorphisms detected in the 240 hepatitis C patients were not significantly different from those in healthy volunteers. The success scores of the Taqman assay were 96.4–100% and those of direct sequencing were 95.8–100%. Univariate analyses of 35 polymorphisms revealed that a *TYK2* exon8 15560-G/T polymorphism (rs2304256) was significantly associated with virologic response to IFN-based therapy [$P$ = 0.050, OR = 0.66 (0.44–0.99)] (Table S5).

In contrast to the univariate analysis, however, multiple logistic regression analyses demonstrated that the rs2304256 was not significant ($P$ = 0.675) (Table 4). As a host factor, only a lower degree of liver fibrosis before therapy ($P$ = 0.007) was significantly associated with SVR in the multiple logistic regression model. On the other hand, HCV genotypes 2 and 3 ($P$ = 0.00005) and a lower viral load before therapy ($P$ = 0.027) were both significantly associated with SVR.

### *Genetic polymorphisms associated with the adverse effects of IFN-based therapy*

A total of 132 of 240 (55.0%) patients required either a discontinuation or a dose reduction of IFN or RBV due to the following adverse events: anaemia ($n$ = 50), neutropenia or leucocytopenia ($n$ = 32), thrombocytopenia ($n$ = 17), depression ($n$ = 7), and other causes (malaise, alopecia, and abdominal discomfort). The relationship between baseline characteristics and occurrence of haematologic adverse effects of the IFN plus RBV combination therapy is summarized in Table S6.

To identify the host genetic polymorphisms associated with the haematologic adverse effects of IFN plus RBV therapy, we focused on decreases in blood cell counts during the therapy and analysed the association with the SNPs in IFN signalling pathway-related genes. Consistent with previous reports [30,31], leucocyte, neutrophil, and platelet counts and haemoglobin levels usually declined in the initial 2–4 weeks of treatment, then stabilized during treatment, and returned to baseline levels within 12 weeks from the end of treatment in patients receiving IFN plus RBV therapy (Fig. 1). Therefore, we evaluated the decreases in leucocyte, neutrophil, and platelet counts and haemoglobin level at 4 weeks of treatment. We first examined the predictive factors for neutropenia. In 240 patients, absolute neutrophil counts decreased by an average of 39.3% from baseline during the first 4 weeks of treatment. Univariate analyses of 32 polymorphisms and clinical features showed that two SNPs, an *IFNAR1* intron2 10848-A/G polymorphism (rs2243594), and a *STAT2* intron5 4757-G/T, were associated with neutropenia caused by IFN-based therapy [$P$ = 0.038, $P$ = 0.020] (Table 5, Table S7). Furthermore, multivariate linear regression analysis confirmed that both polymorphisms were significantly associated with the neutropenia ($P$ = 0.013, $P$ = 0.009). Next, we examined the predictive factors for leucocytopenia. Absolute leucocyte counts decreased by an average of 29.9% from baseline within the first 4 weeks of treatment. Univariate analyses indicated that an *IFNAR1* intron2 10848-A/G polymorphism (rs2243594), an *IRF2* intron6 66675-C/T polymorphism (rs2241500), and female sex were associated with leucocytopenia ($P$ = 0.048, $P$ = 0.026, $P$ = 0.016,

**Table 4** Univariate and multiple logistic regression analyses of SNPs and clinical factors associated with the efficacy of IFN plus ribavirin combination therapy

| | Univariate analysis | | Multiple logistic regression analysis | |
|---|---|---|---|---|
| Variable | *P*-value | OR (95% CI) | *P*-value | OR (95% CI) |
| SNPs | | | | |
| TYK2 15660-G/T | **0.050** | 0.66 (0.44–0.99) | 0.675 | 0.48 (0.14–1.67) |
| Clinical variables | | | | |
| Sex | **0.011** | 2.07 (1.17–3.66) | 0.082 | 2.09 (0.90–4.84) |
| Age | **0.023** | 0.16 (0.04–0.65) | 0.347 | 0.69 (0.11–4.22) |
| HCV genotype | **0.00000002** | 11.6 (5.09–26.6) | **0.00005** | 7.35 (2.54–21.2) |
| Viral load | **0.004** | 0.25 (0.10–0.62) | **0.027** | 0.22 (0.06–0.88) |
| Fibrosis stage | **0.0005** | 12.0 (2.63–54.8) | **0.007** | 10.3 (1.72–62.3) |

*P*-values in boldface are significant. SNP, single nucleotide polymorphism.

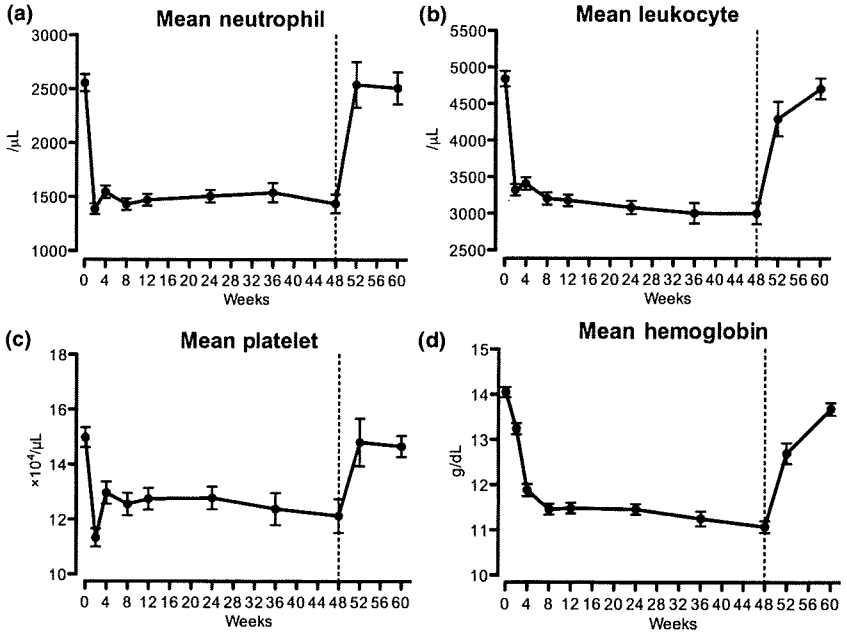


**Fig. 1** Change in mean neutrophil (a), leucocyte (b), and platelet counts (c), and haemoglobin levels (d) during and after IFN plus RBV therapy. The results are shown as mean ± SEM.

respectively). Multivariate analysis, however, indicated that none of the factors, including *IFNAR1* rs2243594, *IRF2* rs2241500 and sex, were significant. Third, we examined the predictive factors for thrombocytopenia. Absolute platelet counts decreased by an average of 12.5% from baseline during the first 4 weeks of treatment. Univariate analyses showed that only an *IRF7* exon2 789-G/A (rs1061501) was associated with thrombocytopenia ($P = 0.031$). Finally, we examined the predictive factors for anaemia. Absolute haemoglobin concentration decreased by an average of 15.8% of baseline within the first 4 weeks of treatment. Univariate analyses revealed that anaemia was associated with older age ($P = 0.0004$), but not with any of the polymorphisms.

We examined the genotype results (variant allele carrier) of an *IFNAR1* intron2 10848-A/G polymorphism (rs2243594), a *STAT2* intron5 4757-G/T polymorphism, and an *IRF7* exon2 789-G/A polymorphism (rs1061501) for their association with various clinical and histologic features among 240 patients (Table S8). None of the factors, however, were associated with the SNPs identified.

## DISCUSSION

In this study, we evaluated the influence of genetic polymorphisms on adverse effects and efficacy of IFN plus RBV combination therapy. Although several studies have evaluated the influence of host genetic polymorphisms on virologic response to IFN-based therapy, no studies have looked at possible association of adverse effects of the IFN-based therapy and host genetic polymorphisms. We report for the first time that certain SNPs in the IFN signalling pathway-related genes were associated with haematologic adverse effects in chronic hepatitis C patients undergoing IFN-based therapy.

**Table 5** Univariate and multiple linear regression analyses of SNPs and clinical factors associated with leucocytopenia, neutropenia and thrombocytopenia

| Variable | Unit of B coefficient | Univariate analysis | | | Multiple analysis | | |
|---|---|---|---|---|---|---|---|
| | | *P*-value | *B* coefficient | SE *B* | *P*-value | *B* coefficient | SE *B* |
| Neutropenia | | | | | | | |
| IFNAR1 10848-A/G | % | **0.038** | 6.94 | 3.31 | **0.013** | 6.43 | 2.57 |
| STAT2 4757-G/T | % | **0.020** | −14.3 | 6.09 | **0.011** | −13.8 | 5.41 |
| Leucocytopenia | | | | | | | |
| IFNAR1 10848-A/G | % | **0.048** | 4.14 | 2.08 | 0.109 | 1.62 | 1.61 |
| IRF2 66675-C/T | % | **0.026** | 3.44 | 1.53 | 0.054 | 3.00 | 1.54 |
| Sex | % | **0.016** | 7.79 | 3.20 | 0.134 | 3.30 | 2.20 |
| Thrombocytopenia | | | | | | | |
| IRF7 789-G/A | % | **0.031** | 4.15 | 1.92 | ND | ND | ND |
| Anaemia | | | | | | | |
| Age | %/year | **0.0004** | 0.28 | 0.08 | ND | ND | ND |

*P*-values in boldface are significant. SE, standard error. ND, not done because only one factor was significant in the univariate analysis.

The representative side effect of IFN-based combination therapy with RBV that causes poor therapeutic tolerance is haematologic toxicity, such as anaemia, neutropenia, and thrombocytopenia [4,32]. In fact, several studies reported that less than half the patients with hepatitis C were able to complete IFN plus RBV combination therapy at the assigned dose of both drugs, causing reduced therapeutic efficacy [5,6]. One thing to be noted is that the decrease in neutrophil and platelet counts induced by IFN-based therapy varies among patients, and thus it is difficult to predict the risk of haematologic toxicities in chronic hepatitis C patients receiving IFN-based therapy. The molecular mechanism of IFN-induced haematologic toxicities, however, is unknown. Several studies suggested the possibility that IFN treatment causes bone marrow suppression [33,34]. In agreement with this hypothesis, it was shown that a significant drop in platelet count after the initiation of IFN therapy is accompanied by a moderate increase in thrombopoietin levels in the failing liver, which may be insufficient to counteract the myelosuppressive action of IFN [35]. Another study suggested that IFN-mediated cytopenia may be due to rapid sequestration of platelets and leucocytes in the capillary beds of the liver and spleen [36]. Our current findings suggest that some of the IFN signalling pathway-related genes are involved in the decrease in neutrophil and platelet counts in response to IFN treatment. Interestingly, a recent study demonstrated that an intrinsic program for apoptosis controls platelet survival and dictates life span [37]. They revealed that platelets are genetically programmed to die by apoptosis and the antagonistic balance between antiapoptotic and proapoptotic molecules determines platelet life span. It is well known that IFN signalling induces the expression of multiple IFN-stimulated genes including molecules with proapoptotic or antiapoptotic function, such as tumour necrosis factor-related apoptosis-inducing ligand Fas, and X-linked inhibitor of apoptosis-associated factor 1 [38]. Thus, it is possible that IFNAR1, STAT2, and IRF7 contribute to the occurrence of neutropenia and thrombocytopenia by regulating the magnitude of IFN signalling involved in the apoptotic pathway in the haematopoietic cells in patients receiving IFN-based treatment.

In this study, three SNPs were associated with cytopenia in chronic hepatitis C patients receiving IFN plus RBV combination therapy. Among them, rs1061501 in the *IRF7* gene was located in the exon region but is a synonymous SNP. Recently, Kimchi-Sarfaty *et al.* demonstrated [39] that a synonymous SNP that did not affect amino acid sequence was capable of changing the function of the resultant protein. Indeed, the presence of a rare codon marked by a synonymous SNP in the *Multidrug Resistance 1* gene affects the timing of cotranslational folding and thereby alters the structure of substrate. Thus, it is possible that the synonymous rs1061501 contributes to a functional change in the IRF7 protein. On the other hand, rs2243594 in the *IFNAR1* gene and the SNP in the *STAT2* gene associated with neutropenia were located in an intronic region. In general, intronic SNPs provide little evidence for changes in protein structure or function, but an intronic mutation in the *p53* gene could have functional consequences by regulating gene expression, suggesting that the effect is mediated by a nonsynonymous and disruptive coding change in linkage disequilibrium with the associated intronic SNP or by a change in RNA splicing, editing, or expression [40]. Thus, it is possible that two intronic SNPs associated with neutropenia contribute to functional changes in the IFNAR1 and STAT2 proteins.

In contrast to the adverse effects of IFN plus RBV combination therapy, none of the host genetic polymorphisms in the IFN signalling pathway-related genes analysed were

associated with therapeutic efficacy. The results indicated that viral factors, including viral genotype and pre-treatment viral load, and histological fibrosis grade were likely to have critical roles in treatment response. Consistent with many previous reports [41–43], we found that HCV genotypes 2 and 3, low viral load, and early fibrosis stage predict a favourable virologic response to IFN plus RBV combination therapy. On the other hand, it was reported that several SNPs in certain genes are associated with efficacy in IFN-based therapy [8–14,16,17]. Many of these previous studies, however, evaluated the association between the SNP and the treatment response using only univariate and not multivariate analyses that included viral factors. In fact, in our univariate analysis, one *TYK2* SNP (rs2304256) showed a possible association with therapeutic efficacy. Multivariate analysis, however, revealed that this SNP was not significant. Taken together, these findings suggest that the viral factors and host histological grade of liver fibrosis are important predictors of the treatment response in chronic hepatitis C infection. Although no significant association was observed between the efficacy and the IFN signalling pathway-related genes examined, it is possible that polymorphisms of other genes might play a role in the treatment response to IFN-based therapy.

In conclusion, we demonstrated that the SNPs in the *IFNAR1* and *STAT2* genes were associated with neutropenia and the SNP in the *IRF7* gene was associated with thrombocytopenia in chronic hepatitis C patients receiving IFN plus RBV combination therapy. In contrast, the virologic factors and histological grade of liver fibrosis are important predictors for virologic response to the IFN-based therapy, whereas no host genetic polymorphisms in IFN signalling pathway-related genes analysed affected the therapeutic efficacy. Further analyses are required to clarify the mechanisms of how those polymorphisms affect the biologic function of the IFN signalling and contribute to the occurrence of haematological adverse effects in IFN-treated patients.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Table S1** Oligonucleotide sequences for primers and probes used for Taqman SNP genotyping assay.

**Table S2** Assay ID of primers and probes used for Taqman SNP genotyping assay.

**Table S3** Oligonucleotide sequences for primers used for PCR amplification and sequencing.

**Table S4** List of discovered polymorphisms in 12 IFN-signalling related genes.

**Table S5** Genotype frequency in the genotyped 35 polymorphisms of the IFN-signalling related genes.

**Table S6** Demographic, virological and clinical features of patients with chronic hepatitis C treated by IFN plus ribavirin combination therapy.

**Table S7** Linear regression analyses of 35 SNPs and clinical factors associated with haematologic adverse effects.

**Table S8** Demographic, and clinical features according to three polymorphisms significantly associated with IFN-induced neutropenia and thrombocytopenia.

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# Absence of Association between *COL1A1* Polymorphisms and High Myopia in the Japanese Population

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**Purpose.** The collagen type I alpha 1 (*COL1A1*) gene was recently reported to be associated with high myopia in the Japanese population. To validate this positive association, the tag single-nucleotide polymorphism (tSNP) approach was used.

**Methods.** Eight tSNPs, including rs2075555 and rs2269336 (previously reported to be high myopia–susceptible SNPs in the Japanese), were selected to tag the linkage disequilibrium blocks harboring the *COL1A1*. These tSNPs were genotyped by using an SNP assay. A total of 427 unrelated Japanese cases with high myopia (axial length, ≥26.50 mm in both eyes; the refraction of the 644 phakic eyes ranged from −5.0 to −36.0 D, with a mean ± SD of −13.61 ± 4.20 D) and 420 Japanese control subjects were recruited. Genotype and allele distributions were compared between the cases and controls by using the $\chi^2$ test, with multiple testing corrections performed by the permutation test.

**Results.** There was no association noted between high myopia and rs2075555 ($P = 0.47$, $P_c > 0.99$) and rs2269336 ($P = 0.40$, $P_c > 0.99$). Meta-analysis of a previous Japanese study and new data obtained in a fixed-effect model indicated a mild significant association of high myopia with rs2075555 (odds ratio [OR], 1.19; 95% confidence interval [CI], 1.03–1.38, $P = 0.022$) and rs2269336 (OR, 1.18; 95% CI, 1.02–1.36, $P = 0.026$). No significant associations were seen with further tSNPs tests.

**Conclusions.** This study did not replicate the previously reported positive association between *COL1A1* and high myopia in the Japanese population, and thus the genetic risk associated with this gene, if any, is weaker than originally reported. (*Invest Ophthalmol Vis Sci.* 2009;50:544–550) DOI:10.1167/iovs.08-2425

Myopia is a common ocular disorder that is found worldwide. The most important contributor to myopic refraction is the axial length of the eyeball (i.e., longer eyes are more myopic),[1–3] and when the elongation of the eyeball is excessive, the condition is called high myopia. It is well known that high myopia is associated with many ocular complications[4] and is one of the major causes of blindness in many developed countries.[5–10] Thus, the economic and social burden of high myopia is an important public health problem.

Recent population-based studies have estimated the prevalence of high myopia in the elderly population to be approximately 1% to 5%,[2,11–16] and this prevalence has been increasing worldwide, especially in the younger East Asian population.[17–19] One possible explanation for the increase in high myopia in developed countries is a change in lifestyle. It has been reported that environmental factors such as near work and higher education can contribute to the development of high myopia. However, genetic factors also have been reported to be responsible for the development of high myopia[20] (for detailed review, see Refs. 21, 22). For example, several twin studies have shown that there is a high heritability of refraction and axial length.[23–28] There have been many studies in which investigators have attempted to use a genetic approach to identify the susceptible locus or genes for high myopia (for detailed review, see Refs. 22, 29, 30), with several genes now reported to have an association.[31–39] However, there are other studies in which the original findings for these genes were not replicated.[25,38,40–48]

Many animal studies on myopia have indicated that there is a local control mechanism of eye growth; hyperopic defocus produces signals from the retina through the retinal pigment epithelium and choroid to cause remodeling of the scleral tissue, and the secondary scleral remodeling results in axial elongation (for detailed review, see Refs. 21, 22, 49, 50). In mammals, the scleral tissue contains approximately 90% collagen by weight, predominantly type I[51] (Zorn M, et al. *IOVS* 1992;33:ARVO Abstract 1811; Norton TT, et al. *IOVS* 1995;36: ARVO Abstract 3517). Several animal studies have reported that mRNA expression of type I collagen in the sclera is reduced during the development of myopia.[52,53] The *COL1A1* (collagen type I, alpha 1) gene encodes the pro-α1 chains of type I collagen. This *COL1A1* is located on 17q21.33, where a myopia susceptibility locus (MYP5, 17q21-22) has been reported.[54] These pathologic, expression, and genetic studies indicated that *COL1A1* is a good candidate gene for myopia. In 2007, Inamori et al.[36] reported that the single-nucleotide polymorphisms (SNPs) rs2075555 and rs2269336 in *COL1A1* are significantly associated with high myopia in the Japanese population. However, Liang et al.[44] reported that the polymor-

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phisms of *COL1A1* are not significantly associated with high myopia in the Taiwanese population.

In the present study, we conducted a systematic case-control study to validate the association between the polymorphisms of the *COL1A1* gene (including previously reported susceptible SNPs) and high myopia in the Japanese population.

## METHODS

All investigations in this study adhered to the tenets of the Declaration of Helsinki. The Institutional Review Board and the Ethics Committee of the each institute approved the protocols of this study. All the patients were fully informed of the purpose and procedures of this study, and written consent was received from each patient.

### Study Population

A total of 427 unrelated Japanese patients with high myopia (mean age ± SD, 57.6 ± 14.1 years; men/women, 31.4% vs. 68.6%) were recruited from the Center for Macular Diseases of Kyoto University Hospital, Fukushima Medical University Hospital, and the high myopia clinic of Tokyo Medical and Dental University Hospital. All underwent comprehensive ophthalmic examinations, including dilated indirect and contact lens slit lamp biomicroscopy, automatic objective refraction evaluation, and measurement of the axial length by applanation A-scan ultrasound (UD-6000; Tomey, Nagoya, Japan) or partial coherence interferometry (IOLMaster; Carl Zeiss Meditec, Dublin, CA). To be enrolled in the study, the patients with high myopia were required to have an axial length of ≥26.50 mm in both eyes. The axial lengths of the 854 eyes ranged from 26.50 to 36.32 mm (mean ± SD, 29.18 ± 1.68). Among the 854 eyes enrolled, 644 (75.4%) were phakic, 185 (21.7%) were pseudophakic, and 25 (2.9%) were aphakic. The mean refraction of the 644 phakic eyes ranged from −5.00 to −36.00 D (mean ± SD, −13.61 ± 4.20). To check the results in another axial length-based definition of high myopia, a subset with longer axial lengths was also defined. The inclusion criterion for this subset was axial length ≥ 28.00 mm in both eyes. A total of 278 patients were enrolled in this subset. The axial length of the 556 eyes in this subset was 29.95 ± 1.43 mm. There were 394 phakic eyes in this subset, with the refraction ranging from −7.25 to −36.00 D (−15.03 ± 4.14). If subjects had preexisting ocular diseases or a history of ocular surgery, with the exception of cataract surgery, they were excluded from the study.

As a population-based control, DNA samples from 420 subjects (mean age ± SD, 44.3 ± 12.1 years; men/women, 46.2% vs. 53.8%) were randomly selected from the Pharma SNP Consortium. The cohort had been recruited for previous genomic studies and was regarded as being representative of the general Japanese population.[55] All participants were Japanese and none of the subjects had any history of ocular diseases.

### SNP Selection and Genotyping

To replicate the positive association of the SNPs with high myopia that has been reported in a previous Japanese study, we genotyped rs2075555 from intron 11 of the *COL1A1*, and rs2269336 from the 5′ upstream region of the *COL1A1*. The associated functions for these two SNPs have yet to be elucidated. To systematically examine the possible association between the polymorphisms of the *COL1A1* gene and the high myopic cases, we used the tag SNP (tSNP) approach. The public dbSNP database build 126 and the HapMap database phase 2 release 22 were used to extract the relevant sequencing information for the *COL1A1* gene and the genotyping information for the SNPs. Haplotypes and linkage disequilibrium (LD) blocks were inferred by a solid spine of LD with a minimum D′ of 0.8, according to Haploview version 4.0.[56] We selected eight tSNPs to tag the LD blocks harbored within and surrounding the *COL1A1* gene (Fig. 1A). Tagging of the LD blocks was based on the software Tagger (http://www.broad.mit.edu/mpg/tagger/ provided in the public domain by the Broad Institute, Massachusetts of Technology, Cambridge, MA), which used a minimum $r^2$ of 0.8 and a minimum minor allele frequency (MAF) of 20% in the Japanese population of the HapMap dataset. It has been reported in a Japanese study that two SNPs (rs2075555 and rs2269336) are high myopia-susceptible polymorphisms.[36] These SNPs were both included within the eight tSNPs. Genomic DNA was extracted from the leukocytes of the peripheral blood and purified (QuickGene-810; Fujifilm, Tokyo, Japan). All the tSNPs were genotyped with an SNP assay (*Taq*man; Applied Biosystems, Foster City, CA), according to the manufacturer's instruction.

### Statistics

The statistical power calculation was performed using the module case-control for discrete traits of the Genetic Power Calculator (http://pngu.mgh.harvard.edu/~purcell/gpc/ provided in the public domain by the Psychiatric and Neurodevelopmental Genetics Unit, Massachusetts General Hospital, Harvard Medical School, Boston, MA).[57] For the calculation, the type 1 error rate was set at 0.05 and the prevalence of high myopia in the general population was set at 1%. The HWE for the genotype distributions was examined by using the $\chi^2$ test in each group. Differences in the observed genotype and allelic frequencies between the cases with high myopia and the control subjects were also examined by the $\chi^2$ test. For the current experiment, we combined our results for the single SNP analysis of rs2075555 and rs2269336 with the results of a previous Japanese study,[36] in which the Mantel-Haenszel method based on the fixed-effect model was used to elucidate their predisposing effects on high myopia in a larger Japanese population. We performed the meta-analysis using the R software package Meta (http://cran.r-project.org/web/packages/rmeta/index.html/ provided in the public domain by The Comprehensive R Archive Network, hosted by the Department of Statistics and Mathematics, University of Vienna, Austria).

Differences in the estimated haplotype frequencies between the cases and the controls were also examined by the $\chi^2$ test. These SNP and haplotype analyses were performed with Haploview ver. 4.0. The multiple testing correction for $P$ ($P_c$) was performed by the permutation test (number of iterations, 10,000), also in Haploview, ver. 4.0. The level of statistical significance was set at $P < 0.05$ and $P_c < 0.05$.

## RESULTS

The distribution of the genotypes for the eight tSNPs among the cases with high myopia and the control subjects were all in HWE ($P > 0.05$). The results of the genotyping for rs2075555 and rs2269336 in the cases with high myopia and the control subjects are shown in Table 1. In this study, there were no significant differences noted for the genotype and allelic frequencies for these two SNPs in the *COL1A1* gene between the patient and the control cases. The results of the meta-analysis for rs2075555 and rs2269336 are shown in Table 2. The Mantel-Haenszel method showed the summary odds ratio (OR) to be 1.19 (95% confidence interval [CI], 1.03–1.38; $P = 0.022$) for rs2075555 and 1.18 (95% CI, 1.02–1.36; $P = 0.026$) for rs2269336, respectively. When we performed the subset analysis on the 278 cases with the longer axial lengths (≥28.00 mm in the both eyes), no new significant differences were found for rs2075555 and rs2269336 in our own study (data not shown). The summary OR for the meta-analysis using the subset was 1.27 (95% CI, 1.08–1.48; $P = 0.0035$) for rs2075555 and 1.25 (95% CI, 1.07–1.46; $P = 0.0051$) for rs2269336, respectively.

We also performed a systematic tSNP approach to assess the possible association between the *COL1A1* and high myopia in Japanese. The distributions of the allelic frequencies for all the eight tSNPs are given in Table 3. None of the eight tSNPs showed significant differences between the cases with high myopia and the control subjects with regard to the distribution of the genotype and allelic frequency. We also performed a

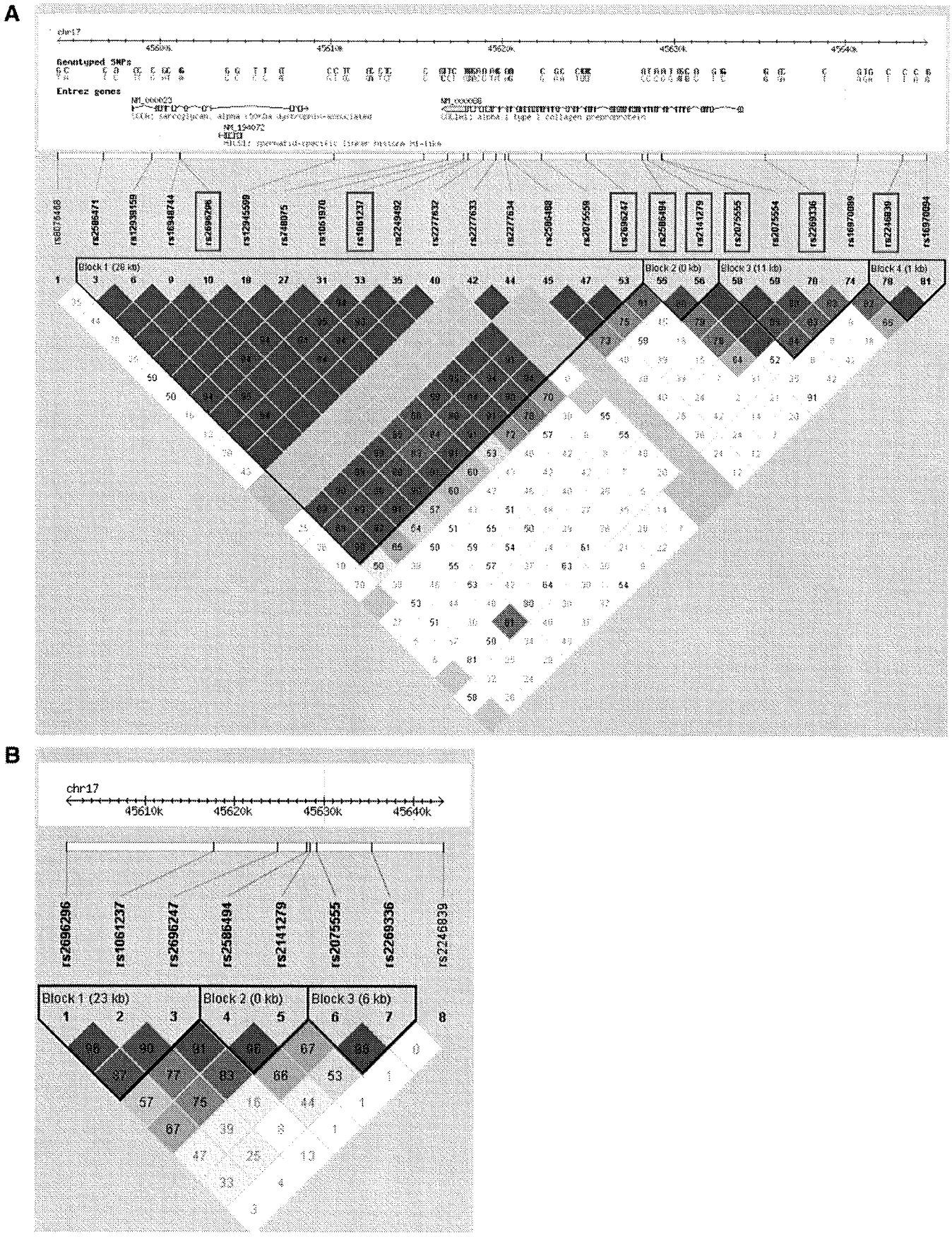


**FIGURE 1.** LD structure across the *COL1A1* region and selected tag SNPs. LD blocks were inferred by a solid spine of LD with a minimum D′ of 0.8. **(A)** LD structure in Japanese samples from the HapMap database. SNPs with MAF > 5% are displayed, with the selected 8 tSNPs shown in boxes. **(B)** LD structure for the samples obtained in the present study (427 unrelated Japanese cases with high myopia [axial length ≥ 26.50 mm in both eyes] and 420 healthy Japanese controls). Three haplotype blocks were identified. The distribution of the haplotypes from each of the three blocks is shown in Table 4.

**TABLE 1.** Frequencies of Genotypes and Alleles of rs2075555 and rs2269336 in the Current Study

| | Genotype | | | | | Allele | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| SNP ID* | | Case (%) | Control (%) | P† | OR (95% CI) | | Case (%) | Control (%) | P† | OR (95% CI) |
| rs2075555 | CC | 167 (39.2) | 158 (37.7) | 0.736 | 1.17 (0.79–1.75) | C | 528 (62.0) | 505 (60.3) | 0.471 | 1.07 (0.88–1.31) |
| | CA | 194 (45.5) | 189 (45.1) | | 1.14 (0.77–1.68) | A | 324 (38.0) | 333 (39.7) | | |
| | AA | 65 (15.3) | 72 (17.2) | | 1.00 (ref.) | | | | | |
| rs2269336 | CC | 141 (33.8) | 125 (29.9) | 0.444 | 1.14 (0.78–1.67) | C | 469 (56.2) | 453 (54.2) | 0.400 | 1.09 (0.90–1.32) |
| | CG | 187 (44.8) | 203 (48.6) | | 0.93 (0.65–1.33) | G | 365 (43.8) | 383 (45.8) | | |
| | GG | 89 (21.3) | 90 (21.5) | | 1.00 (ref.) | | | | | |

The nucleotides were defined on the forward strand of the reference sequence by dbSNP Build 126.

* SNP ID in National Center for Biotechnology Information (NCBI; Bethesda, MD) dbSNP Build 126.

† The nominal probabilities were calculated by the $\chi^2$ test.

subset analysis on cases with axial lengths ≥ 28.00 mm. However, no new significant differences were found for the subjects in our study (data not shown).

We identified three haplotype blocks in the *COL1A1* gene (Fig. 1B). The estimated haplotype frequencies in the cases with high myopia and the control subjects are shown in Table 4. The haplotype frequencies were not significantly different between the patients with high myopia and the control subjects after the multiple testing corrections. Before correction, only one haplotype in block 1 showed a trend for a mildly significant difference in distribution ($P = 0.014$, $P_c = 0.14$). However, a haplotype analysis using the subset with axial lengths ≥ 28.00 mm did not show significant results for any of the blocks, even before correction (data not shown).

In addition, to check the results of our analyses using the same inclusion criteria that were used in a previous Japanese study,[36] we performed another subset analysis on 261 binocular phakic cases with refractions < −9.25 D (mean refraction ± SD, −14.46 ± 3.94 D; mean axial length ± SD, 29.17 ± 1.60 mm). The allelic frequency distributions for all the eight tSNPs in the subset analysis are given in Table 5. No new significant differences were noted for the genotype and allelic frequencies for rs2075555 and rs2269336. A haplotype in block 1 (the same haplotype described above) showed a trend for a mildly significant different distribution ($P = 0.034$, $P_c = 0.33$). However, there were no tSNPs or haplotypes that showed any significant differences after the multiple testing corrections.

## DISCUSSION

The results in this study did not show significant associations with high myopia of the two SNPs of the *COL1A1* gene (rs2075555 and rs2269336, which have been reported to be high-myopia-susceptible SNPs in the Japanese population[36]). A systematic examination using the tSNP approach to access the possible association between the *COL1A1* gene and Japanese high myopia also did not find any significant results. The power calculation results that were based on the multiplicative model showed that our own observations rejected the reported ORs of rs2075555 (OR, 1.36) and rs2269336 (OR, 1.31) from the previous Japanese study with 85.9% and 78.7% power, respectively.

In our study, we defined high myopia by axial length instead of refraction. On the other hand, in the previous Japanese study that was included in our current analyses, they defined high myopia as refraction < −9.25 D.[36] Thus, one possible explanation for the discrepancy that was observed between the previous Japanese study and our own observations might be related to the difference in the way that high myopia was defined. To further examine this possibility, we performed a subset analysis on binocular phakic cases that had refraction < −9.25 D in both eyes, and we found no further significant differences in the present study. High myopia is most commonly defined by refraction. However, corneal curvature and the intraocular lens may also affect the refraction. Among these multiple factors, the axial length is the most important contributor to myopic refraction.[1–3] Hence, we suggest that the axial length is a more appropriate parameter than refraction when assessing the association between the *COL1A1* gene and high myopia. However, our study could not show any significant result whether the axial length or the refraction was chosen as the parameter. We cannot conclude which of the two, axial length or refraction, is the more appropriate parameter to assess the association between the *COL1A1* gene and high myopia.

**TABLE 2.** Meta-analysis* of the *COL1A1* rs2075555 and rs2269336 in Japanese Subjects with High Myopia

| | | Case | | Control | | | |
|---|---|---|---|---|---|---|---|
| SNP ID† | Source | Subject Number | Risk Allele‡ *n* (%) | Subjects (*n*) | Risk Allele‡ *n* (%) | OR (95% CI) | P§ |
| rs2075555 | Current study | 426 | 528 (62.0) | 419 | 505 (60.3) | 1.07 (0.88–1.31) | 0.47 |
| | Inamori et al.[36] | 328 | 422 (64.3) | 326 | 372 (57.1) | 1.36 (1.09–1.70) | 0.0071 |
| | Total | 754 | | 745 | | 1.19 (1.03–1.38) | 0.022 |
| rs2269336 | Current study | 417 | 469 (56.2) | 418 | 453 (54.2) | 1.09 (0.90–1.32) | 0.40 |
| | Inamori et al.[36] | 329 | 397 (60.3) | 330 | 354 (53.6) | 1.31 (1.06–1.64) | 0.014 |
| | Total | 746 | | 748 | | 1.18 (1.02–1.36) | 0.026 |

* This meta-analysis was performed using the Mantel-Haenszel method based on the fixed-effect model.

† SNP ID in NCBI dbSNP Build 126.

‡ Risk alleles of rs2075555 (A/C) and rs2269336 (C/G) are allele C and allele C, respectively. The nucleotides were defined on the forward strand of the reference sequence by the dbSNP Build 126. We confirmed through personal communication that the nucleotides for rs2269336 (C/G) in a previous study by Inamori et al.[36] were defined on the reverse strand of the reference sequence by dbSNP.

§ The nominal probabilities were calculated by the $\chi^2$ test.

TABLE 3. Association of Eight Tagged SNPs of the *COL1A1* with High Myopia in the Current Study

| SNP ID* | Position† | Ref‡ | Var‡ | Case–Control | | OR (95% CI) | P§ | |
|---|---|---|---|---|---|---|---|---|
| | | | | Allele Counts | Allele Frequencies | | Nominal | Corrected |
| rs2696296 | 45601230 | G | A | 427:421, 395:445 | 0.504, 0.470 | 1.14 (0.94–1.38) | 0.171 | 0.886 |
| rs1061237 | 45617774 | T | C | 469:377, 477:339 | 0.554, 0.585 | 0.88 (0.73–1.07) | 0.214 | 0.933 |
| rs2696247 | 45624902 | A | G | 589:257, 567:271 | 0.696, 0.677 | 1.10 (0.89–1.35) | 0.386 | 0.997 |
| rs2586494 | 45628154 | A | C | 407:441, 425:409 | 0.480, 0.510 | 0.89 (0.73–1.08) | 0.224 | 0.940 |
| rs2141279 | 45628463 | T | C | 270:574, 268:568 | 0.320, 0.321 | 1.00 (0.81–1.22) | 0.977 | 1.000 |
| rs2075555 | 45629290 | A | C | 324:528, 333:505 | 0.380, 0.397 | 0.93 (0.77–1.13) | 0.471 | 1.000 |
| rs2269336 | 45635355 | C | G | 469:365, 453:383 | 0.562, 0.542 | 1.09 (0.90–1.32) | 0.400 | 0.998 |
| rs2246839 | 45643395 | C | T | 321:527, 321:511 | 0.379, 0.386 | 0.97 (0.80–1.18) | 0.759 | 1.000 |

Axial length ≥ 26.5 mm in both eyes.

* SNP ID in NCBI dbSNP Build 126.

† Position of the polymorphism in the reference sequence NT_010783.14.

‡ Ref and Var were the reference and variant nucleotides, respectively, that were defined on the forward strand of the reference sequence by dbSNP.

§ The nominal probabilities were calculated by the $\chi^2$ test, and the multiple testing corrections were performed by the permutation test (number of iterations = 10,000).

Another difference between the previous study and our own observations is that we used a population-based control. The prevalence of high myopia in the general population has been estimated to be approximately 1% to 5% in elderly adults.[2,11–16] Even if the control subjects in our study had no history of ocular diseases, the possibility exists that some of the eyes might have had an axial length ≥ 26.50 mm without the presence of vision threatening complications. If this were the case, this would be a possible explanation for the negative results that we found for our case-control association study. To check the results for a different axial-length-based definition, we also performed a subset analysis on cases with longer axial lengths (≥28.00 mm in both of the eyes). However, no new significant differences were found in the present study. Further subset analyses by redefining the cutoff value of axial length (27.00, 27.50, 28.50, and 29.00 mm) did not show any significant results (data not shown). Thus, we can conclude that the results of the present study did not replicate the previously reported Japanese study, which found significant associations for rs2075555 and rs2269336 with high myopia.

The results of our meta-analysis suggested that there were mildly significant associations between these two SNPs and high myopia in the Japanese population. However, it should be noted that we combined the data of our own study with the data of a previous Japanese study, a study that was the first to report positive results.[36] There was a potential for publication bias in the first positive study, and indeed, the reported ORs in the first positive studies were higher than most of the results that have been reported for subsequent replication studies.[58] Therefore, actual ORs of these SNPs are estimated at up to the ORs that are suggested by the results of the meta-analysis in this study. We concluded that the genetic risk in the *COL1A1* gene, if any, is weaker than has been originally reported.

In conclusion, the present study failed to replicate the positive association between the polymorphisms of the *COL1A1* gene and high myopia that has been reported in a prior study involving Japanese subjects. To elucidate whether the *COL1A1* gene in the MYP5 locus is associated with high myopia in the Japanese population, additional genetic and molecular biological studies are needed.

TABLE 4. Association of Haplotypes across the *COL1A1* Region with High Myopia in the Current Study

| Haplotype* | Frequency | Case–Control | | OR (95% CI) | P† | |
|---|---|---|---|---|---|---|
| | | Ratio Counts | Frequencies | | Nominal | Corrected |
| Block 1 | | | | | | |
| ATA | 0.489 | 403.5:450.5, 424.3:415.7 | 0.472, 0.505 | 0.88 (0.73–1.06) | 0.179 | 0.889 |
| GCG | 0.293 | 243.0:611.0, 253.0:587.0 | 0.285, 0.301 | 0.92 (0.75–1.14) | 0.452 | 0.998 |
| GCA | 0.132 | 130.0:724.0, 93.7:746.3 | 0.152, 0.112 | 1.43 (1.08–1.90) | 0.014 | 0.142 |
| GTA | 0.062 | 57.3:796.7, 48.3:791.7 | 0.067, 0.057 | 1.18 (0.79–1.75) | 0.411 | 0.997 |
| ATG | 0.017 | 11.8:842.2, 17.3:822.7 | 0.014, 0.021 | 0.67 (0.32–1.41) | 0.288 | 0.980 |
| Block 2 | | | | | | |
| AC | 0.488 | 405.6:448.4, 420.6:419.4 | 0.475, 0.501 | 0.90 (0.75–1.09) | 0.289 | 0.980 |
| CT | 0.315 | 269.6:584.4, 263.3:576.7 | 0.316, 0.313 | 1.01 (0.82–1.24) | 0.922 | 1.000 |
| CC | 0.192 | 175.9:678.1, 150.2:689.8 | 0.206, 0.179 | 1.19 (0.94–1.52) | 0.156 | 0.842 |
| Block 3 | | | | | | |
| CC | 0.545 | 474.3:379.7, 448.3:391.7 | 0.555, 0.534 | 1.09 (0.90–1.32) | 0.369 | 0.993 |
| AG | 0.381 | 319.3:534.7, 326.4:513.6 | 0.374, 0.389 | 0.94 (0.77–1.14) | 0.533 | 0.999 |
| CG | 0.067 | 54.8:799.2, 57.9:782.1 | 0.064, 0.069 | 0.93 (0.63–1.36) | 0.699 | 1.000 |

Axial length ≥ 26.5 mm in both eyes. The nucleotides were defined on the forward strand of the reference sequence by dbSNP Build 126.

* Haplotypes and linkage disequilibrium (LD) blocks were inferred by a solid spine of LD with a minimum D′ of 0.8. The LD structure for the Japanese samples in the current study is shown in Figure 1(B).

† The nominal probabilities were calculated by the $\chi^2$ test, with the multiple testing corrections performed by the permutation test (no. of iterations = 10,000).

**TABLE 5.** Association of Eight Tagged SNPs of *COL1A1* with a Subset of High Myopia in the Current Study

| | | | | Case–Control | | | *P*§ | |
|---|---|---|---|---|---|---|---|---|
| **SNP ID*** | **Position†** | **Ref‡** | **Var‡** | **Allele Frequencies** | **Allele Counts** | **OR (95% CI)** | **Nominal** | **Corrected** |
| rs2696296 | 45601230 | G | A | 266:250, 395:445 | 0.516, 0.470 | 1.20 (0.96–1.49) | 0.105 | 0.743 |
| rs1061237 | 45617774 | T | C | 277:239, 477:339 | 0.537, 0.585 | 0.82 (0.66–1.03) | 0.087 | 0.640 |
| rs2696247 | 45624902 | A | G | 348:168, 567:271 | 0.674, 0.677 | 0.99 (0.78–1.25) | 0.933 | 1.000 |
| rs2586494 | 45628154 | A | C | 259:259, 425:409 | 0.500, 0.510 | 0.96 (0.77–1.20) | 0.732 | 1.000 |
| rs2141279 | 45628463 | T | C | 158:358, 268:568 | 0.306, 0.321 | 0.94 (0.74–1.19) | 0.581 | 1.000 |
| rs2075555 | 45629290 | A | C | 197:323, 333:505 | 0.379, 0.397 | 0.92 (0.74–1.16) | 0.496 | 1.000 |
| rs2269336 | 45635355 | C | G | 296:218, 453:383 | 0.576, 0.542 | 1.15 (0.92–1.43) | 0.222 | 0.956 |
| rs2246839 | 45643395 | C | T | 197:323, 321:511 | 0.379, 0.386 | 0.97 (0.77–1.22) | 0.798 | 1.000 |

Eyes were binocular phakic and had refraction $< -9.25$ D.

* SNP ID in NCBI dbSNP Build 126.

† Position of the polymorphism in the reference sequence NT_010783.14.

‡ Ref and Var are, respectively, reference and variant nucleotides defined on the forward strand of the reference sequence by dbSNP.

§ The nominal probabilities were calculated by the $\chi^2$ test, and the multiple testing corrections were performed by the permutation test (number of iterations = 10,000).

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ORIGINAL ARTICLE

# Diffusion-weighted magnetic resonance imaging in autoimmune pancreatitis

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**Abstract**
*Purpose.* The aim of this study was to investigate the usefulness of diffusion-weighted magnetic resonance imaging (DWI MRI) for the diagnosis and evaluation of autoimmune pancreatitis (AIP).
*Materials and methods.* A total of 4 consecutive patients with AIP, 5 patients with chronic alcoholic pancreatitis (CP), and 13 patients without pancreatic disease (controls) were studied. DWI was performed in the axial plane with spin-echo echo-planar imaging single-shot sequence. Apparent diffusion coefficients (ADCs) were measured in circular regions of interest in the pancreas. In AIP patients, abdominal MRI was performed before, and 2–4 weeks after steroid treatment. Follow-up study was performed chronologically for up to 11 months in two patients. The correlation between ADCs of the pancreas and the immunoglobulin G4 (IgG4) index (serum IgG4 value/serum IgG4 value before steroid treatment) was evaluated.
*Results.* In the AIP patients, DWI of the pancreas showed high signal intensity, and the ADCs of the pancreas (mean ± SD: $0.97 \pm 0.18 \times 10^{-3}$ mm$^2$/s) were significantly lower than those in patients with CP ($1.45 \pm 0.10 \times 10^{-3}$ mm$^2$/s) or the controls ($1.45 \pm 0.16 \times 10^{-3}$ mm$^2$/s) (Mann-Whitney U-test, $P < 0.05$). In one AIP patient with focal swelling of the pancreas head that appeared to be a mass, DWI showed high signal intensity throughout the pancreas, indicating diffuse involvement. The ADCs of the pancreas and IgG4 index were significantly inversely correlated (Spearman's rank correlation coefficient, $r_s = -0.80$, $P < 0.05$).
*Conclusion.* Autoimmune pancreatitis showed high signal intensity on DWI, which improved after steroid treatment. ADCs reflected disease activity. Thus, diffusion-weighted MRI might be useful for diagnosing AIP, determining the affected area, and evaluating the effect of treatment.

**Key words** Autoimmune pancreatitis · Diffusion-weighted magnetic resonance imaging · Steroid treatment

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## Introduction

Autoimmune pancreatitis (AIP) is a special form of chronic pancreatitis characterized by extensive fibrosis with lymphocyte and plasmacyte infiltration in the exocrine pancreas and responsiveness to steroid treatment. The peak age of AIP onset is during the sixth decade, and men are predominantly affected. The patients usually have no or only slight symptoms and often present with obstructive jaundice. Sclerosing cholangitis, sialoadenitis, and retroperitoneal fibrosis are sometimes associated with AIP.[1–3] Serum immunoglobulin G4 (IgG4) is useful for diagnosing AIP; and changes in serum IgG4 levels reflect disease activity.[4] The characteristics of AIP in imaging studies are diffuse or focal hypoechoic swelling of the pancreas and diffuse or focal irregular narrowing of the main pancreatic duct.[1,2,5] Characteristic findings

on dynamic computed tomography (CT) are delayed enhancement of the pancreas and a capsule-like rim. On magnetic resonance imaging (MRI), the pancreatic parenchyma typically shows low signal intensity on fat-suppressed T1-weighted images and high signal intensity in T2-weighted images.[6–8] The findings of diffusion weighted MR imaging (DWI) in AIP, however, have not been reported. We investigated the usefulness of DWI for the diagnosis and evaluation of disease activity in AIP.

## Materials and methods

### Patients

The hospital ethics committee approved this retrospective study. Four consecutive patients with AIP (four men with a mean ± SD age of 64.5 ± 9.4 years), five patients with chronic alcoholic pancreatitis (CP) (five men, 64.8 ± 4.9 years), and thirteen patients without pancreatic disease (nine men, four women, 59.4 ± 14.4 years), as controls, were studied retrospectively. The diagnosis of AIP was based on the diagnostic criteria of AIP reported by the Japan Pancreas Society[9] as follows.

- Pancreatic image examinations show narrowing of the main pancreatic duct and enlargement of the pancreas, which are characteristic of the disease.
- Laboratory data indicate the presence of autoantibodies or elevated levels of serum gamma globulin, IgG, or IgG4.
- Histopathological examinations of the pancreas show fibrosis and pronounced infiltration of cells (mainly lymphocytes and plasmacytes), which is called lymphoplasmacytic sclerosing pancreatitis.

For a diagnosis of AIP, the first criterion must be present together with the second or third criterion. It is necessary to exclude malignant diseases, such as pancreatic or biliary cancers.

On CT, two of the four patients with AIP showed diffuse swelling of the pancreas, and the other two showed focal swelling of the pancreas head. The mean ± SD serum IgG4 on admission was 508.5 ± 248.1 mg/dl (range 298–831 mg/dl; normal <105 mg/dl). Following a diagnosis of AIP, 30–40 mg prednisolone was administered daily for 2–4 weeks, after which the dose was tapered.

### MR imaging protocols

A Gyroscan Intera Master (1.5 T; Philips Medical Systems, Best, The Netherlands) was used for MRI. Diffusion-weighted MRI was performed in the axial plane with a spin-echo echo-planar imaging single-shot sequence [repetition time (TR) 2883 ms, echo time (TE) 86 ms, flip angle 90°], b values of 0 and 1000 s/mm$^2$ with a four-channel sense body coil. A respiratory trigger was not used; the scan was performed under free-breathing conditions.[10] Fifty slices were produced with a 4-mm slice thickness and a 1-mm interslice gap. The other parameters were field of view (FOV) 360 mm, matrix 128 × 128, double number of samples averaged (NSA) sense factor 3.0. An apparent diffusion coefficient (ADC) map was obtained for each slice position.[11]

### Assessment

The ADCs were measured in a circular region of interest (ROI) within the pancreas from ADC maps on the workstation (AW4.2; GE Healthcare, Milwaukee, WI, USA). Three ROIs were placed on the head, body, and tail of the pancreas; and average ADCs were calculated. The largest possible ROI was used for each portion. In cases of atrophy of the parenchyma in the tail, ADCs of the head and body were averaged.

In AIP patients, abdominal MRI was performed prior to initiating steroid treatment and 2–4 weeks after the initiation of steroid treatment. A follow-up study was performed chronologically for up to 11 months in two patients. ADCs of the pancreas of the four AIP patients prior to steroid treatment were compared with those of patients with CP and controls. A Mann-Whitney U-test was used to compare ADCs among the three groups. $P < 0.05$ was considered statistically significant.

### Correlation between ADCs and AIP disease activity

As an indicator of AIP disease activity, we defined the IgG4 index as the serum IgG4 value divided by the IgG4 value obtained before initiating the steroid treatment. The correlation between the ADCs of the pancreas and IgG4 index was evaluated by Spearman's rank correlation coefficient. $P < 0.05$ was considered statistically significant.

## Results

Findings from imaging studies of the four AIP patients are summarized in Table 1. In patients 1, 2, and 3, DWI of the diffuse pancreas showed high signal intensity (Figs. 1, 2). Notably, in patient 3, although focal swelling of the pancreas head appeared as a mass on enhanced CT, DWI showed high signal intensity in the entire pancreas (Fig. 2). In patient 4, who also showed focal

swelling of the pancreas head, DWI showed high signal intensity in the pancreas head.

The mean ± SD ADCs of the pancreas in patients with AIP ($0.97 \pm 0.18 \times 10^{-3}$ mm$^2$/s) were significantly lower than the ADCs of patients with CP ($1.45 \pm 0.10 \times 10^{-3}$ mm$^2$/s) or the controls ($1.45 \pm 0.16 \times 10^{-3}$ mm$^2$/s) ($P < 0.01$) (Fig. 3).

In AIP patients, the signal intensity decreased after steroid treatment (Figs. 1, 2). The ADCs of the pancreas increased after steroid treatment (Fig. 4). The ADCs of the pancreas and the IgG4 index were significantly inversely correlated ($r_s = -0.80$, $P < 0.05$) (Fig. 5).

## Discussion

Diffusion weighted imaging showed high signal intensity for AIP before treatment with steroids and improvement after steroid therapy. AIP could be clearly differentiated from chronic alcoholic pancreas and normal controls on DWI,

The DWI method reflects the random thermal motion of molecules.[12] We speculate that high signal intensity in patients with AIP might reflect severe inflammation with lymphocytes and plasmacytes, and a decrease in the signal intensity after steroid treatment might reflect amelioration of these pathological changes. Because the number of patients studied was small, more studies with larger numbers of patients are required to confirm these preliminary results.

Early, precise evaluation of the effect of steroid treatment is important in patients with AIP. The significant correlation between ADCs of the pancreas and the IgG4 index observed in the present study indicates that ADCs reflect disease activity. Therefore, DWI might be useful for evaluating the effects of steroid treatment and for follow-up study.

Pancreatic carcinoma also shows high signal intensity in DWI.[13] Therefore, a differential diagnosis should be carefully performed with other diagnostic modalities, including endoscopic retrograde cholangiopancreatography, especially in cases of focal swelling of the pancreas.[14,15] In one AIP patient (case 3) with prominent swelling of the pancreas head that appeared to be a mass, DWI showed high signal intensity throughout the pancreas, indicating diffuse involvement. Upstream pancreatitis was less likely because dilatation of the main

**Table 1.** Findings on imaging studies in four patients with autoimmune pancreatitis before treatment

| Patient no. | Age (years)/sex | Swelling[a] | Signal intensity on DWI |
|---|---|---|---|
| 1 | 63/M | Diffuse | High/diffuse |
| 2 | 53/M | Diffuse | High/diffuse |
| 3 | 65/M | Focal | High/diffuse |
| 4 | 77/M | Focal | High/focal |

DWI, diffusion-weighted magnetic resonance imaging
[a] Swelling of the pancreas seen on computed tomography

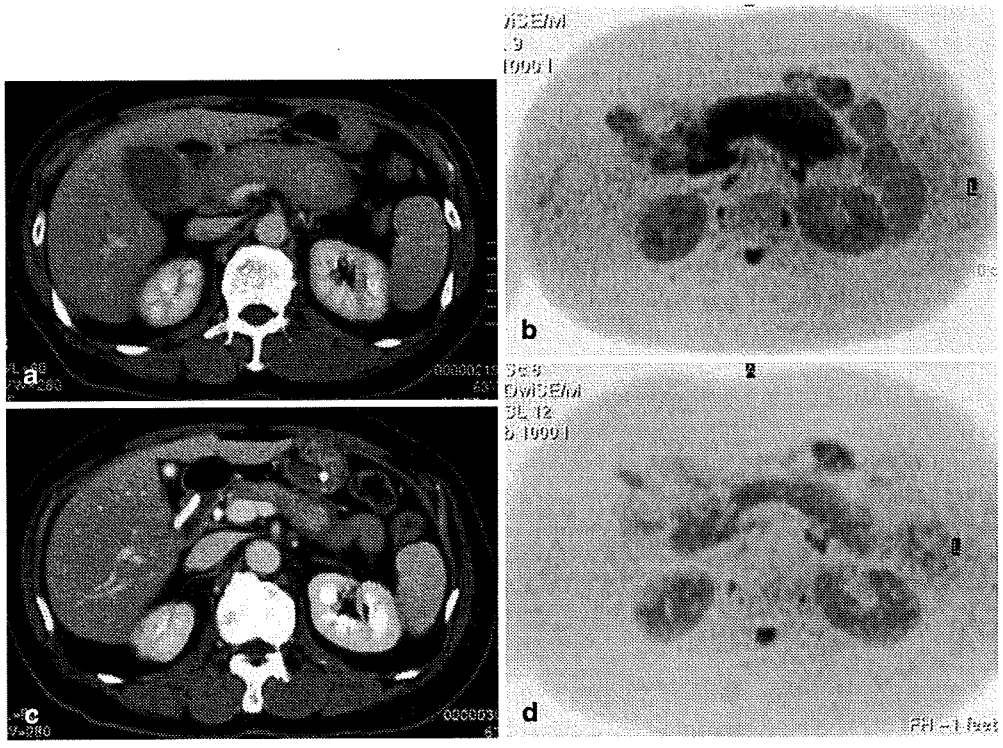



**Fig. 1.** A 63-year-old man had autoimmune pancreatitis. **a** Dynamic computed tomography (CT) of the abdomen (delayed phase) on admission. Diffuse swelling of the pancreas with delayed enhancement and a capsule-like rim at the tail were observed. **b** Diffusion-weighted magnetic resonance imaging (DWI) of the pancreas on admission. The pancreas showed a high intensity signal. **c** Dynamic CT of the abdomen (delayed phase) 2 weeks after initiating steroid treatment. The pancreatic swelling was significantly reduced. **d** DWI of the pancreas 2 weeks after initiating steroid treatment. The signal intensity had decreased

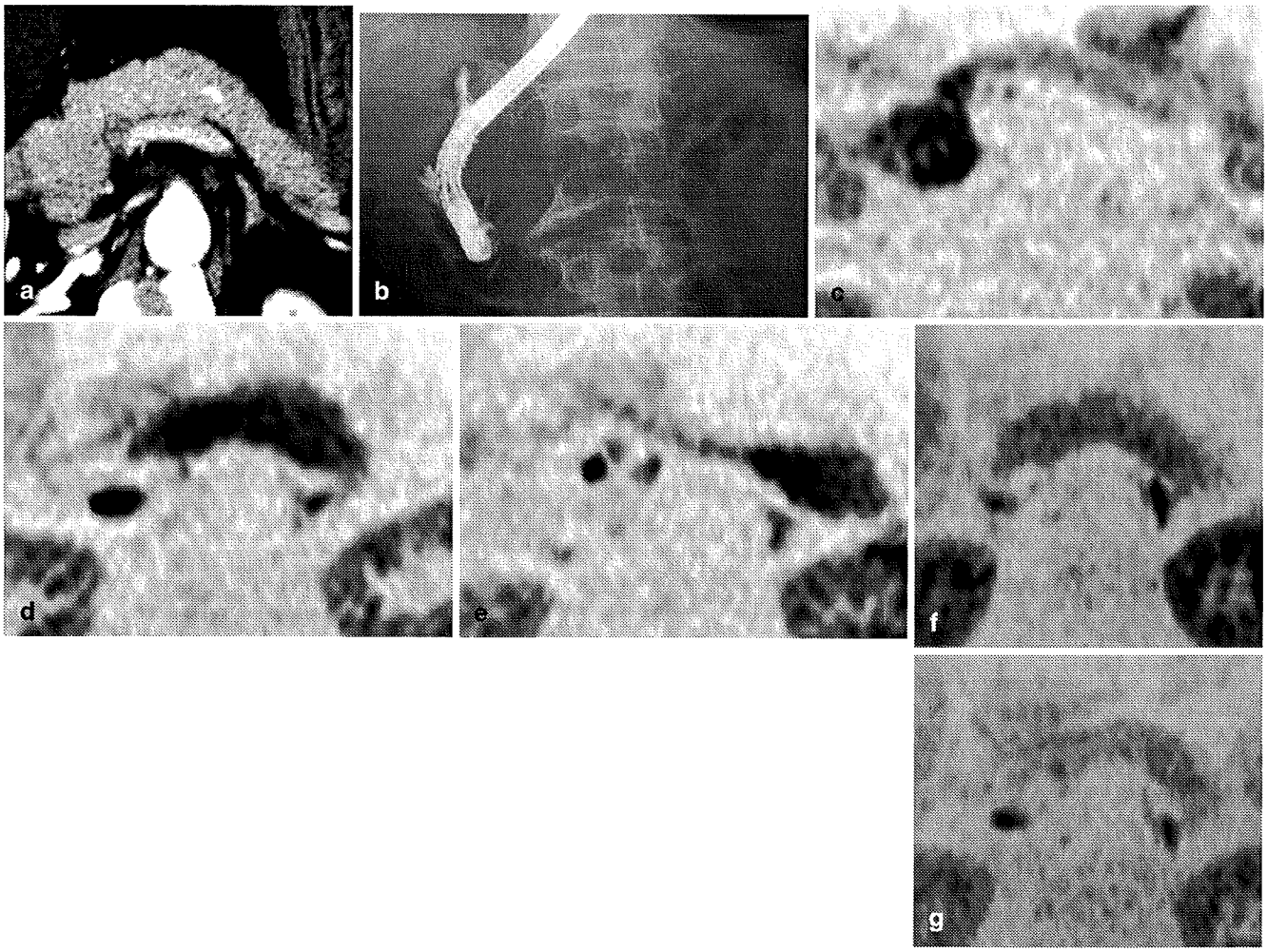
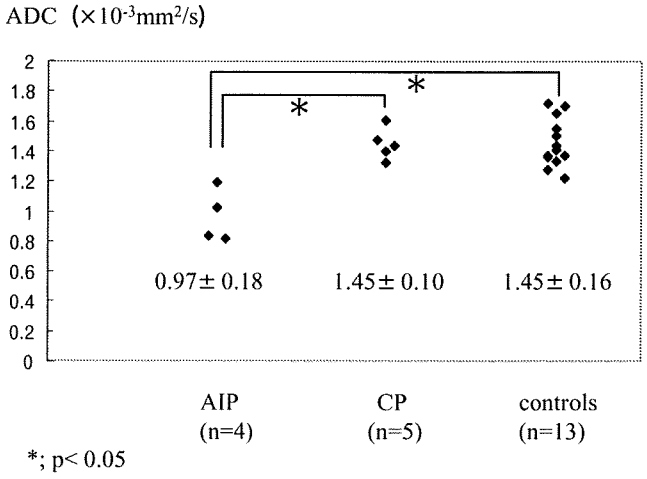



**Fig. 2.** A 65-year-old man had autoimmune pancreatitis. **a** Dynamic computed tomography of the abdomen (early-phase) on admission. **b** Endoscopic retrograde pancreatography on admission. There was irregular narrowing of the main pancreatic duct (MPD) in the head and irregularity of the MPD in the body and tail. The MPD was not dilated. **c** DWI of the pancreas head on admission. The pancreas showed a high intensity signal. **d** DWI of the pancreas body on admission. **e** DWI of the pancreas tail on admission. **f** DWI of the pancreas body 4 weeks after initiating steroid treatment. The signal intensity had decreased. **g** DWI of the pancreas body 11 months after initiating steroid treatment. There was a further decrease in the signal intensity

**Fig. 3.** Apparent diffusion coefficients (*ADC*) of the pancreas in patients with autoimmune pancreatitis (*AIP*), patients with chronic alcoholic pancreatitis (*CP*), and controls. The ADCs of the pancreas in patients with AIP were significantly lower than the ADCs of patients with CP or the controls



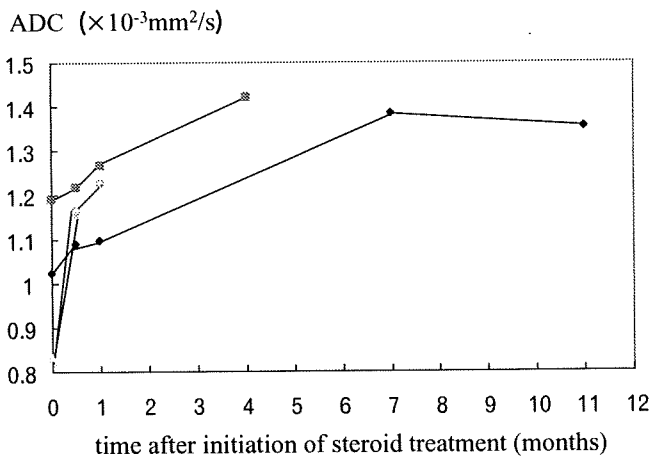



**Fig. 4.** Chronological changes in the ADC of the pancreas before and after initiation of steroid treatment in patients with AIP. The ADC of the pancreas increased after steroid treatment

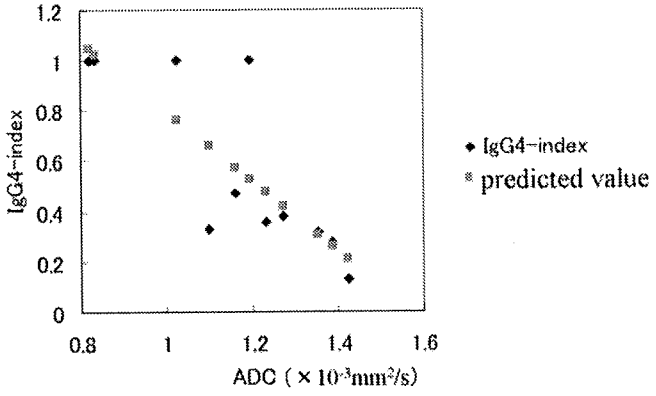



**Fig. 5.** Correlation between ADCs of the pancreas and the immunoglobulin G4 (IgG4) index (serum IgG4 value divided by the IgG4 value before steroid treatment) of patients with AIP. The ADCs of the pancreas and the IgG4 index were significantly inversely correlated ($r_s = -0.80$, $P < 0.05$)

pancreatic duct was not prominent on endoscopic retrograde cholangiopancreatography (Fig. 2B). After steroid treatment, the size of the entire pancreas, including the body and tail, decreased.

Thus, DWI was useful for determining the affected area in patients with AIP. DWI can be used to determine diffuse involvement of AIP, which is sometimes difficult to recognize with other imaging modalities; and it might be helpful for differentiating between AIP and pancreatic carcinoma.

## Conclusion

Autoimmune pancreatitis showed high signal intensity in DWI, which improved after steroid treatment. ADCs reflected the disease activity. This modality might be useful for diagnosing AIP, determining the affected area, and evaluating the effect of treatment.

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# Association of *UCP2* and *UCP3* polymorphisms with heart rate variability in Japanese men

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***Objectives*** **The mitochondrial uncoupling proteins UCP2 and UCP3 are implicated in energy metabolism and regulation of reactive oxygen species, which are closely involved in autonomic nervous system function. Heart rate variability (HRV) reflects cardiac autonomic regulation and has been used to evaluate dysfunction of the autonomic nervous system in hypertension and cardiovascular diseases. We examined the association between polymorphisms in the *UCP2* and *UCP3* genes and HRV in healthy young Japanese men.**

***Methods*** **The 45 bp insertion/deletion polymorphism in exon8 of *UCP2* and the −55C/T polymorphism in the *UCP3* promoter region were genotyped ($n = 255$). Cardiac autonomic function was evaluated by power spectral analysis of HRV during supine rest and in a standing position. Low-frequency (<0.15 Hz) and high-frequency (>0.15 Hz) components of HRV were quantified by frequency domain analysis.**

***Results*** **The I/I genotype of the *UCP2* 45 bp insertion/deletion polymorphism was associated with relatively higher blood pressure and HRV sympathetic indices (low frequency percentage and low frequency:high frequency ratio) at supine rest. For the −55C/T polymorphism of *UCP3*, individuals carrying the −55T allele had significantly lower HRV sympathetic indices, but higher HRV parasympathetic indices (high frequency and high frequency percentage), than carriers of the C/C genotype at standing. Both *UCP2* and *UCP3* polymorphisms were significantly associated with a third-degree family history of hypertension, diabetes, and obesity. Additionally, carriers of the *UCP2* 45 bp I allele −*UCP3* −55C/C combined genotype had the lowest HRV sympathetic and the highest HRV parasympathetic indices at standing among the combined genotypes.**

***Conclusion*** ***UCP2*** **and *UCP3* polymorphisms were associated with HRV in young and healthy states, suggesting a significant relationship between autonomic cardiovascular regulation and UCP2/UCP3 polymorphisms.** *J Hypertens* **27:305–313 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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**Abbreviations:** ACR, urinary albumin-to-creatinine ratio; AI, augmentation index; AIc, aortic (central arterial) augmentation index; AIp, peripheral augmentation index; APOGH, African Project on Genes on Hypertension; BMI, body mass index; BP, blood pressure; DBP, diastolic blood pressure; DM, diabetes mellitus; E/A, early-to-late transmitral velocity; $HbA_{1c}$, glycated haemoglobin; LV, left ventricular; LVM, left ventricular mass; LVMI, left ventricular mass indexed to height$^{2.7}$; MWT, mean wall thickness; PWV, carotid-femoral pulse wave velocity; SBP, systolic blood pressure; TOD, target organ changes

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## Introduction

Uncoupling proteins (UCPs) are a family of mitochondrial transporters that can uncouple oxidative phosphorylation through a dissipation of the proton gradient across the inner mitochondrial membrane. Apart from the roles of both UCPs in energy and fatty acid metabolism [1,2], UCP2 and UCP3 are also involved in preventing the formation of reactive oxygen species (ROS) [3,4]. ROS are produced by the mitochondria during mitochondrial respiration, and lowering of the proton gradient across the inner mitochondrial membrane by UCPs results in a reduction in ROS generation [3,4]. Indeed, superoxide or ROS production was increased in macrophages, pancreatic islets, or skeletal muscle mitochondria from UCP2-deficient or UCP3-deficient mice, respectively [5–7].

UCP2 is widely distributed in human tissues (e.g. adipose tissue, skeletal muscle, heart, placenta, liver, kidney, pancreas, and brain), and UCP3 is predominantly expressed in the skeletal muscle and the heart, whereas both UCPs are also expressed in the central nervous system (CNS) or dorsal root ganglion [8,9]. It has been shown that UCP2 plays neuromodulatory/neuroprotective roles in the CNS, and UCP3 protects neurons from glucose-induced degeneration by preventing ROS

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formation [8,9]. Recent studies have demonstrated that ROS have a sympathoexcitatory effect at the central, cardiac, and peripheral tissues [10,11]. These data suggest a possible association between UCPs and autonomic nervous system (ANS) activity through ROS regulation.

Many genetic polymorphisms have been identified in the *UCP2* and *UCP3* genes (http://www.ncbi.nlm.nih.gov/projects/SNP/). Of these, a 45 bp insertion/deletion polymorphism (rs1800795) in the 3′ untranslated region (UTR) of exon 8 in *UCP2* and a −55C/T polymorphism (rs1800849) in the *UCP3* promoter region have been intensively studied in association with various pathological phenotypes [12,13]. The biological or functional relevance of both polymorphisms of *UCPs* have been previously shown. The *UCP2* 45 bp insertion/deletion polymorphism was reported to be related to metabolic rate [14] and increased BMI [15–17], whereas the *UCP3* −55T allele was associated with increased expression of *UCP3* mRNA in the skeletal muscle [18]. However, the (patho)physiological impact of both polymorphisms remains unknown.

Heart rate variability (HRV) analysis provides useful indirect markers of cardiac autonomic modulation and is an early and sensitive indicator of the pathological states of hypertension or cardiovascular disease (CVD) [19–22]. Impairment of autonomic balance is closely involved in the pathophysiology of hypertension and CVD [23,24]. Using HRV analysis, sympathovagal imbalance, especially sympathetic predominance or decreased vagal activity, was often observed in patients with hypertension or CVD [25–28], whereas some HRV indices could predict the future onset of these diseases [20,21] and cardiac or all-cause mortality after myocardial infarction [29–32]. Recent reports suggest that heritable variations may play critical roles in ANS function as reflected in HRV [33–35]. Although UCP2 and UCP3 are involved in the ANS either directly by effects on autonomic pathway or indirectly through ROS regulation, the contribution of genetic polymorphisms of *UCPs* to ANS function remains unclear. Thus, in the present study, we assessed the association of *UCP2* 45 bp insertion/deletion and *UCP3* −55C/T polymorphisms with cardiac ANS by HRV analysis in young Japanese men.

## Methods

### Participants

Two hundred and fifty-five young, healthy Japanese men, recruited at random from Kyoto University, participated in each examination after written informed consent was obtained. The ages of participants ranged from 18 to 28 years (21.5 ± 0.1 years). All participants were normotensive [causal supine blood pressure (BP) <140/90 mmHg] and nonobese (BMI <30 kg/m$^2$). It was determined by interview that participants were not taking any medication and had no history of organic diseases such as CVD, metabolic disorder, renal disease, or neuropathy. BMI, BP [systolic BP (SBP) and diastolic BP (DBP)], and heart rate (HR) (at supine rest and standing) were measured as baseline characteristics, and family history (including whether participants had relatives within the third degree who had hypertension, diabetes, or obesity) was investigated by interviews. All participants underwent ECG recording and power spectral analysis of HRV. However, HRV could not be determined for three participants in the supine position, 10 participants in the standing position, and for one participant in both postures. The study protocol was reviewed by the appropriate institutional review committee of Kyoto University Graduate School of Human and Environmental Studies, and the guidelines of the Declaration of Helsinki were followed.

### Genotyping

Genomic DNA was extracted from whole blood (DNA Extractor WB Kit; Wako, Osaka, Japan) or buccal cells (BuccalAmpTM DNA Extraction Kit; EPICENTRE Biotechnologies, Madison, Wisconsin, USA). *UCP2* 45 bp insertion/deletion and *UCP3* −55C/T polymorphisms were genotyped as previously described [14,36]. The genotype of the *UCP2* 45 bp insertion/deletion polymorphism of one participant could not be determined because of an inadequate sample of buccal cells.

### ECG R–R interval power spectral analysis

Details of the HRV analysis methodology used have been well reviewed [37,38]. Each participant was studied in a quiet room at an ambient temperature of 25°C. Participants rested in a supine position for at least 20 min before ECG recording. CM5 lead ECG was continuously recorded during supine rest and postural change to a standing position. After 10 min of supine rest, participants stood up by the bedside and remained at standing rest for another 10 min. During the test, the respiratory rate was controlled at 0.25 Hz (15 breaths/min) by means of an electric metronome to reduce significant variations in HRV spectral powers resulting from individual variations in breathing frequency and to avoid interference with the low-frequency component by the parasympathetic component [39]. The R–R interval power spectral analysis procedures have been described previously [34,35]. Briefly, ECG R–R interval data obtained from the CM5 lead were digitized at 1000 Hz, and the derived R–R interval time series were then aligned in a 2 Hz sequence for power spectral analysis. Direct current component and linear trend were completely eliminated by digital filtering for a band-pass between 0.007 and 0.5 Hz. After passing through the Hamming-type data window, power spectral analysis was performed using a fast Fourier transform on the consecutive 480-s time series of R–R interval data obtained during the tests. Very low frequency (VLF, 0.007–0.035 Hz), low frequency (0.035–0.15 Hz), high frequency (0.15–0.5 Hz),

and total power (0.007–0.5 Hz) were evaluated by integrating the spectrum for the respective bandwidth. Low-frequency and high-frequency powers were expressed in both absolute units ($ms^2$) and in normalized units (%). The normalized low-frequency or high-frequency powers were calculated as follows: low-frequency power (%) = (low-frequency/total power − VLF) × 100 and high-frequency power (%) = (high-frequency/total power − VLF) × 100 [25]. The ratio of low-frequency to high-frequency powers (low-frequency power/high-frequency power) was also calculated as an index of sympathetic modulation [37,38]. The average HR in beats per minute in each position (supine rest/standing) was derived from the R waves of the ECG.

### Statistical analysis

Hardy–Weinberg equilibrium was verified by comparison of the observed and expected genotype frequency using the $\chi^2$ test. Linkage disequilibrium analysis was performed with the Haploview program [40]. In line with previous studies [25,37], a natural logarithmic transformation was used to normalize the distribution of HRV power spectral indices, as these data showed a distribution skewed to the right. Differences in clinical characteristics and log-transformed values (ln) of HRV indices were evaluated by Student's *t*-test or one-way analysis of variance with Bonferroni's method for post-hoc multiple comparisons, whenever appropriate. For comparison of HRV indices, adjusted *P* values were also provided after adjustment of potential confounding factors (age, BMI, and family history of hypertension, diabetes, or obesity). Data were expressed as mean ± standard error of mean. The $\chi^2$ test was performed for analysis of the relationship of genotype distributions with family history of hypertension, diabetes, or obesity. Statistical analysis was performed using the Statview Statistical Package (SAS Institute Inc.; Cary, North Carolina, USA). Significant differences were considered to be present at a *P* value of less than 0.05.

## Results

### Characteristics of study participants

The general clinical characteristics of study participants are shown in Table 1. All participants were in good health with almost ideal BMI and BP. Frequencies of the *UCP2* 45 bp I allele and the *UCP3* −55T allele were 20.7 and 31.0%, respectively, similar to that reported previously in the Japanese population [41,42]. There were no significant deviations from the Hardy–Weinberg equilibrium for *UCP2* 45 bp insertion/deletion ($\chi^2 = 1.45$, $P = 0.23$) or *UCP3* −55C/T ($\chi^2 = 1.04$, $P = 0.31$) polymorphisms.

The clinical characteristics of study participants classified by the *UCP2* 45 bp insertion/deletion or the *UCP3* −55C/T polymorphisms are shown in Table 2. Carriers of the *UCP2* 45 bp I/I genotype had significantly higher SBP and DBP than the 45 bp D/D or insertion/deletion carriers. A significantly higher third-degree family history of hypertension, diabetes, and obesity were also observed for the 45 bp I allele carriers of *UCP2*. On the contrary, the *UCP3* −55T allele carriers had significantly lower BMI than carriers of the C/C genotype, and body weight also tended to be relatively lower in the −55T allele carriers. Although not statistically significant, the −55T allele carriers tended to have lower mean BP than the C/C carriers. Additionally, a significantly less common family history of hypertension, diabetes, or obesity was seen for carriers of the −55T allele.

**Table 1 Clinical characteristics of study participants ($n = 255$)**

| Characteristics | Mean ± SEM |
|---|---|
| Age (years) | 21.5 ± 0.1 |
| Height (cm) | 172.4 ± 0.3 |
| Body weight (kg) | 62.5 ± 0.5 |
| BMI ($kg/m^2$) | 21.0 ± 0.2 |
| SBP (mmHg) | 111.2 ± 0.6 |
| DBP (mmHg) | 63.0 ± 0.6 |
| MBP (mmHg) | 79.0 ± 0.5 |
| HR (supine rest, bpm; $n = 251$) | 61.0 ± 0.5 |
| HR (standing, bpm; $n = 244$) | 81.4 ± 0.7 |
| Third-degree family history of HT, DM, or obesity (%) | 27.1 |

bpm, beats per minute; DBP, diastolic blood pressure; DM, diabetes mellitus; HR, heart rate; HT, hypertension; MBP, mean blood pressure; SBP, systolic blood pressure; SEM, standard error of mean.

### Association of UCP2 and UCP3 polymorphisms with heart rate variability indices

Power spectral parameters of HRV according to the *UCP2* 45 bp insertion/deletion or the *UCP3* −55C/T polymorphisms are shown in Tables 3 and 4. As described [37,38], power spectral analysis of HRV has generally shown two major distinct regions of periodicity in ECG R–R intervals: a high-frequency component (>0.15 Hz) and a low-frequency component (<0.15 Hz). Previous studies have shown that the high-frequency component is mediated solely by parasympathetic nervous system activity, and that the low-frequency component arises from both sympathetic and parasympathetic nervous activities [37,38]. In addition, the ratio of the low-frequency to the high-frequency component has been considered as an index of sympathovagal balance or sympathetic activity [37,38]. Low-frequency and high-frequency components of HRV were quantified by frequency domain analysis and expressed in absolute and normalized units.

At supine rest, carriers of the *UCP2* 45 bp I/I genotype had significantly higher HRV sympathetic indices (low frequency percentage and low frequency/high frequency) and a lower parasympathetic index (high frequency percentage) than 45 bp insertion/deletion carriers, although there was no significant difference when compared with the 45 bp D/D genotype. For the −55C/T polymorphism of *UCP3*, in a standing position, the *UCP3* −55T allele carriers had a significantly higher high frequency and high frequency percentage than the C/C carriers. On the