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Splicing variant of *WDFY4* augments MDA5 signaling and the risk of clinically amyopathic dermatomyositis

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(Splicing variant of WDFY4 augments MDA5 signaling and the risk of clinically amyopathic dermatomyositis

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ABSTRACT

Objectives Idiopathic inflammatory myopathies (IIM) are a heterogeneous group of rare autoimmune diseases in which both genetic and environmental factors play important roles. To identify genetic factors of IIM including polymyositis (PM), dermatomyositis (DM), and clinically amyopathic DM (CADM), we performed the first genome-wide association study (GWAS) for IIM in an Asian population.

Methods We genotyped and tested 496,819 SNPs for association using 576 IIM patients and 6,270 control subjects. We also examined the causal mechanism of disease associated variants by *in silico* analyses using publicly available data sets as well as by *in vitro* analyses using reporter assays and apoptosis assays.

Results We identified a variant in *WDFY4* that was significantly associated with CADM (rs7919656; odds ratio (OR)=3.87; $P=1.5\times 10^{-8}$). This variant had a cis-splicing quantitative trait locus (QTL) effect for a truncated *WDFY4* isoform (tr-*WDFY4*), with higher expression in the risk allele. Trans-expression QTL (eQTL) analysis of this variant showed a positive correlation with the expression of NF- κ B associated genes. Furthermore, we demonstrated that both *WDFY4* and tr-*WDFY4* interacted with pattern recognition receptors such as TLR3, TLR4, TLR9, and MDA5 and augmented the NF- κ B activation by these receptors. *WDFY4* isoforms also enhanced MDA5-induced apoptosis, to a greater extent in the tr-*WDFY4*-transfected cells.

Conclusions As CADM is characterized by the appearance of anti-MDA5 autoantibodies and severe lung inflammation, the *WDFY4* variant may play a critical role in the pathogenesis of CADM.

INTRODUCTION

Idiopathic inflammatory myopathies (IIM) are autoimmune conditions characterized by muscle weakness and inflammation, and the most common types are polymyositis (PM) and dermatomyositis (DM).^{1 2} Clinically amyopathic DM (CADM), a subset of DM, lacks distinct muscle features but often manifests rapidly progressive interstitial pneumonia (RPIP).³⁻⁶ The mortality rate of CADM at 6 months from diagnosis reaches approximately 50%, making it one of the most fatal autoimmune diseases.^{7 8} The appearance of autoantibodies such as anti-MDA5-antibodies in CADM suggests a role for autoimmunity in the pathogenesis.⁹⁻¹¹ Although the etiology of IIM remains unknown, virus infection may be the major environmental factor.¹² In addition, IIM patients and their close relatives are more likely to develop other autoimmune diseases, suggesting shared genetic factors.¹³⁻¹⁵ In fact, a previous genome-wide association studies (GWAS) and candidate gene analyses in European populations demonstrated multiple loci that were shared with other autoimmune diseases.¹⁵⁻¹⁸

However, when compared with other autoimmune diseases, little is known about the genetic background of IIM. This may be due to the rarity of disease, precluding researchers from designing sufficiently powered studies. In addition, because IIMs are a heterogenous group of rare disorders, an overall pooled analysis might also reduce the power for detecting loci specific to certain subtypes. Therefore, as suggested¹⁹, studies of disease subsets are needed to increase the power for detecting subset-specific genetic components. Here, to elucidate genetic loci associated with IIM, we performed a GWAS for IIM in the Japanese population. This study was the first GWAS for IIM in an Asian population. We also performed a GWAS for CADM as a subset analysis, which was the first such study in the world.

Methods

Subjects

We enrolled 592 IIM cases (mean age, 55.5 ± 14.2 years; female, 74.6%) from 18 medical institutes in Japan. PM/DM fulfilled the definite or probable diagnostic criteria of Bohan and Peter.¹ The diagnosis of CADM was based on the criteria by Sontheimer et al.^{3,6} We enrolled 6270 control subjects without history of autoimmune diseases (mean age, 57.0 ± 13.8 years; female, 48.2%) through the Biobank project²⁰. The European subjects (21 CADM cases) were recruited as part of an international myositis genetics consortium (MYOGEN),¹⁷ and four controls for each case (84 controls) were matched for ethnicity using principal components analysis (PCA) coordinates.²¹ All subjects were provided with written informed consent for participation in the study as approved by the ethical committee of each institutional review board.

GWAS

We genotyped case samples using HumanOmniExpressExome BeadChip and control samples using HumanOmniExpress BeadChip and HumanExome BeadChip (Illumina). For quality control, we excluded single nucleotide polymorphisms (SNPs) with a call rate < 0.99 , a mean allele frequency (MAF) < 0.05 , and those not in Hardy-Weinberg equilibrium in controls ($P < 1 \times 10^{-6}$). We removed samples that showed second-degree relatedness or closer by an identity-by-state method. We performed PCA using four reference populations from HapMap data including CEU, YRI, JPT and CHB using SmartPCA software.²² We identified outliers not belonging to the JPT/CHB cluster by using the top two principal components. After this screening, we selected 576 cases and 6270 controls for subsequent analysis. This sample size had statistical power > 0.5 for

detecting common variants with moderate effects (MAF=0.5 and OR=1.5) as calculated by QUANTO.²³ The European samples were genotyped by the Immunochip (Illumina)¹⁸. We performed genotype imputation within the *WDFY4* locus by Minimac²⁴ using the 1000 Genomes Project Asian data as reference.

eQTL analysis

Both cis- and trans- eQTL analyses were performed using Matrix eQTL with the linear model.²⁵ We used the Geuvadis project data²⁶ of European samples (n=373), “GD462.GeneQuantRPKM.50FN.samplename.resk10.norm.txt” for the gene level eQTL analysis and “GD462.ExonQuantCount.45N.50FN.samplename.resk10.txt” for the exon level eQTL analysis.

wPGSA

To predict the activities of transcriptional regulators responsible for observed gene expression changes resulting from trans-eQTL effects of tr-*WDFY4*, we used weighted parametric gene set analysis (wPGSA)²⁷ with some modifications (supplementary figure S7). ChIP-seq data of multiple types of human cells were obtained from the GEO-database. In total, 3082 ChIP-seq data of 431 TFs were used. We used the trans-eQTL effects of *WDFY4* variant (rs7919656) on every gene (β obtained by Matrix-eQTL) as input, instead of the log-fold change of the gene expression.

Reporter assay

The coding sequences of *WDFY4* and tr-*WDFY4* were cloned into the pcDNA3.1D/V5-His-TOPO vector using cDNAs from EBV-immortalized B cells.

Human pattern recognition receptors were cloned into the pEF-BOS-FLAG vector (TLR3 was provided by Dr. Matsumoto, Hokkaido University; TLR4 and TLR9 were provided by Dr. Miyake, the University of Tokyo). UNC93B1 was cloned into the pcDNA5/FRT/V5-His vector. UNC93B1 was co-transfected for the experiments of TLR7 or TLR9, because it is essential to deliver these receptors to endolysosomes.²⁸ We cultured HEK-293 cells in DMEM supplemented with 10% fetal bovine serum. We transfected *WDFY4* expression vectors, the pGL4.32 vector (NF- κ B-response element and luc2P) and the pGL4.74 vector (hRluc/TK) (Promega) into cells using the X-tremeGENE HP Transfection Reagent (Roche). The p55C1B-Luc vector²⁹ (provided by Dr. Fujita, Kyoto University) was used instead of pGL4.32 for IRF3 reporter assays. After 18h incubation, the cells were stimulated with poly (I:C) (Enzo Life Sciences), LPS (Sigma-Aldrich), imiquimod (InvivoGen), ODN2006 (InvivoGen), or high molecular weight poly (I:C)³⁰ (provided by Dr. Fujita) for 4h. We then collected the cells and measured luciferase activity using Dual-Luciferase Reporter Assays according to the manufacturer's protocol (Promega).

Immunoprecipitation and western blotting

HEK-293 cells were transfected with each *WDFY4* isoform and PRR and cultured for 24h. The cells were lysed in RIPA buffer³¹ and then incubated with Dynabeads protein-G (Life Technologies) coated with anti-FLAG-antibody (Sigma-Aldrich) and incubated at 4°C for 30min. The beads were then washed and boiled in SDS buffer before electrophoresis through NuPAGE Tris-Acetate gels. The proteins were transferred to PDVF membranes (Millipore), which were then probed with anti-V5-antibody (Invitrogen), visualized using ECL reagent (GE Healthcare), and

detected using LAS-3000 (Fuji Film). We also performed immunoprecipitation using the sera from patients, instead of anti-FLAG-antibody.

Apoptosis assay

HepG2 Cells were transfected with each *WDFY4* isoform using the Lipofectamine 3000 reagent (Invitrogen). The same vector with cloned *LacZ* was also transfected as a mock. Cells were incubated for 24h and stimulated with 5µg of poly (I:C). The cells were permeabilized and stained with CaspACE FITC-VAD-FMK (Promega) and anti-His-Alexa 647. Caspase activity was evaluated in the His-positive cells using the BD Accuri C6 flow cytometer (BD Biosciences) to detect apoptotic cells.

Immunofluorescence staining

HEK-293 cells were grown on coverslips and transfected with the vectors for *WDFY4* isoforms and *MDA5*. Cells were fixed in 4% paraformaldehyde, permeabilized in 0.25% Triton X-100, and stained with anti-V5 and anti-mouse IgG-Alexa488 (Invitrogen) for *WDFY4* and anti-FLAG-Cy3 (Sigma-Aldrich) for *MDA5* detection. DAPI was used for counter-staining. Images were acquired with the confocal microscope N-SIM (Nikon) using 100X objective lens. The data were analyzed in 3D-SIM mode by using NIS-Elements software (Nikon).

Measurement of anti-MDA5

We measured anti-MDA5 autoantibodies in the sera from the CADM patients (18 sera were available) by protein-immunoprecipitation.⁹

Statistics

We tested the association of SNPs by allelic test using Plink 1.9,³² setting the genome-wide significance threshold at $\alpha=5\times 10^{-8}$. We used a quantile-quantile plot to evaluate the potential effects of population stratification. We used analysis of variance (ANOVA) with post-hoc Tukey's test in the reporter assays and apoptosis assays.

RESULTS

GWAS for IIM

We genotyped the Japanese samples using Illumina SNP arrays for over 960,000 SNPs. After quality control filtering, the effective sample size was 576 IIM cases and 6,270 controls, and 496,819 SNPs were tested. The genomic inflation factor λ_{GC} was 1.024 (supplementary figure S1), suggesting a negligible effect of population stratification. Cases comprised 236 PM and 340 DM patients including 33 CADM patients, and GWASs for these disease subsets were also performed. No significant association signal ($P<5\times10^{-8}$) was found in any of total IIM, PM or DM (supplementary figure S2). However, when CADM patients were evaluated alone, a significant association was observed for an intronic SNP of *WDFY4* (WDFY Family Member 4; rs7919656; OR=3.87; 95% CI=2.23–6.55; $P=1.5\times10^{-8}$) (figure 1A and table 1). This SNP showed a nominal association with IIM, even when CADM patients were excluded ($P=0.037$). In European CADM patients, although no obvious association signal was observed in rs7919656, distinct association signals ($r^2<0.2$ with rs7919656) were observed for the neighboring variants (rs11101462, $P=0.0092$; rs2889697, $P=0.0058$, supplementary figure S3)

Splicing QTL effect of WDFY4 variant on truncated isoform

To unravel the causal mechanism of the *WDFY4* locus in this disease subset, we first searched for candidate functional variants. Among the variants in strong linkage disequilibrium (LD) with rs7919656 ($r^2>0.8$) in the 1000 genome project Asian samples, there is no coding variant that altered the amino-acid sequences of neighboring genes (supplementary figure S4). We next searched the expression quantitative trait locus

(eQTL) data of lymphoblastoid B-cells provided by the Geuvadis project.²⁶ When correlations of the SNP (rs7919656) genotype and expression of both the gene and exons were evaluated, a significant eQTL effect for an alternative exon of *WDFY4* was found (figure 1B). As no obvious eQTL effect was observed for expression of the whole gene or of neighboring exons (supplementary figure S5) this variant or variants in LD may have a splicing QTL effect for this *WDFY4* isoform. Among such variants, two SNPs in intron 35 (rs10776647/rs10776648; figure 1C and supplementary figure S4) were candidate causal variants in this region; however, they did not disrupt classical splicing associated motifs. The alternative exon extends exon 36 of *WDFY4*, which introduces an alternate stop codon and truncates the protein (figure 1C, D). We verified the existence of this truncated isoform (tr-*WDFY4*) by cloning it with cDNA from EBV-immortalized B cells and expressing it in HEK-293 cells (figure 1E).

Trans eQTL effect of *WDFY4* variant on NF- κ B activity

WDFY4 encodes WDFY family member 4, which has a predicted BEACH domain and five WD domains at the C-terminal side³³ (figure 1D). Although *WDFY4* is expressed in dendritic cells, neutrophils, B-cells and macrophages (supplementary figure S6), to our knowledge, there have been no studies into its protein function. To obtain insight regarding the function of *WDFY4*, we performed trans-eQTL analysis of the variant using the same expression data of lymphoblastoid B-cells. A total of 387 genes were correlated with the number of risk alleles (uncorrected $P < 0.05$), although no gene displayed genome-wide significant correlation (FDR < 0.05). Notably, 279 genes (72.1%) were positively correlated, indicating that upregulated expression of tr-*WDFY4* results in upregulation of these genes. Among the top 10 genes with the largest

trans-eQTL effects (β as calculated in the Matrix eQTL analysis), *TYK2* and *PTPN6* are well known regulators of signal pathways in B-cells (figure 2A). We then sought to identify the transcriptional factors (TFs) present downstream of tr-*WDFY4* by weighted parametric gene set analysis (wPGSA).²⁷ wPGSA utilizes all available ChIP-seq data obtained from multiple types of human cells and predicts the activities of transcriptional regulators responsible for observed gene expression changes (figure 2B and supplementary figure S7). When trans-eQTL effects (β) were used as input, among the 431 TFs evaluated, 6 TFs displayed significant associations (FDR<0.05) (figure 2C and supplementary table S1). We focused on TF65 (*RELA*/p65), because it comprises nuclear factor-kappa B (NF- κ B) heterodimers that are one of the most important TFs in the immune system.³⁴ Based on these findings, we assumed that the NF- κ B pathway is activated downstream of WDFY4. Indeed, NF- κ B reporter assays showed that both WDFY4 and tr-WDFY4 enhanced NF- κ B activity when transfected into HEK-293 cells with stimulation of TNF- α (figure 2D).

WDFY4 augments signals of innate pattern recognition receptors

These observations, as well as a recent report that WDFY1, another member of the WDFY gene family, augmented TLR3 and TLR9 signaling³⁵, motivated us to investigate the role of WDFY4 in PRR signaling. We chose TLR3/TLR4/TLR7/TLR9 and MDA5 for analysis considering their implied roles in disease pathogenesis^{4 36 37}, as well as their potential to activate NF- κ B.³⁸ We first performed NF- κ B reporter assays by simultaneously transfecting each WDFY4 isoform with each PRR. While both WDFY4 isoforms enhanced NF- κ B signaling of TLR3/TLR4/TLR9 and MDA5, they had a negligible effect on TLR7 signaling (figure 3A). Of note, tr-WDFY4 markedly

enhanced the MDA5 signal when stimulated with poly (I:C), a synthetic analog of double-stranded RNA. We also examined the impact of WDFY4 isoforms on cytokine production by quantifying the levels of IL-8 in the cell culture supernatants. Although both isoforms significantly increased the IL-8 production in steady state, the difference to mock transfectant became insignificant when cells were stimulated with poly (I:C) (supplementary figure S8). Next, we examined the physical interaction of WDFY4 isoforms and these PRRs by co-immunoprecipitation. Both V5-tagged WDFY4 isoforms interacted with these FLAG-tagged PRRs to greater or lesser degrees (figure 3B). The strongest interaction was observed between tr-WDFY4 and MDA5, which was consistent with the findings of reporter assays.

MDA5-mediated sensing of virus triggers not only endogenous immune responses but also cellular apoptosis to eliminate virus-infected cells.³⁹ Therefore, to further evaluate the function of WDFY4 in the context of MDA5 signaling, we performed apoptosis assays. We used HepG2 cells for these assays, because stimulation of these cells with poly (I:C) is accompanied by upregulation of MDA5 and results in the induction of apoptosis⁴⁰ (figure 4A). When HepG2 cells were transfected with the WDFY4 isoforms and stimulated with/without poly (I:C), apoptosis was augmented to a greater extent in the tr-WDFY4-transfected cells than in the WDFY4-transfected cells (figure 4B). Again, this result is consistent with the observations regarding these isoforms in the NF- κ B assays. As the apoptosis induced by MDA5 was reported to be independent of type I interferon signals⁴¹, we evaluated the effect of WDFY4 isoforms on type I interferon induction by co-transfected MDA5 using IRF3 reporter assays²⁹. For comparison, we also examined TLR3 signaling. While no effect of WDFY4 isoforms on TLR3 induction of interferon was detected, MDA5 induction of interferon was markedly

repressed when the WDFY4 isoforms were co-transfected with MDA5 (figure 4C).
These findings suggested that the WDFY4 isoforms are able to interact with MDA5 and
alter its signaling in cells. This possibility was further supported by the observations
that transfected WDFY4 isoforms and MDA5 co-localized in the cytoplasm of cells as
assessed using immunofluorescence (figure 5A), and that the anti-MDA5-positive sera
from patients could co-immunoprecipitate tr-WDFY4 proteins (figure 5B).

DISCUSSION

We here demonstrated a significant association between a variant in *WDFY4* and CADM susceptibility. Association of *WDFY4* locus has been previously reported with systemic lupus erythematosus (SLE)⁴² and rheumatoid arthritis.⁴³ A recent study reported that the SLE risk variant (rs2663052) also had a splicing QTL effect on *WDFY4*.⁴⁴ However, rs2663052 is not in tight LD with the CADM risk variant (rs7919656, $r^2=0.46$ in the Asian samples), and rs2663052 displayed a weaker association signal in CADM in the present study ($P=0.0012$). A missense variant (rs7097397), which is in moderate LD with rs7919656 ($r^2=0.58$ in the Asian samples), may be another candidate causal variant in SLE⁴², but the association in CADM was moderate ($P=0.0022$).

The fact that the CADM risk variant was also associated with upregulated expression of tr-*WDFY4* suggested that *WDFY4* was a strong candidate gene in this locus. Although the biological function of *WDFY4* was unknown, our analysis by wPGSA utilizing the trans-eQTL effects of *WDFY4* variant implied that NF- κ B was involved in the downstream regulation of *WDFY4*. Indeed, as demonstrated by *in vitro* assays, tr-*WDFY4* augmented the NF- κ B signals induced by PRRs including MDA5. Our *in vitro* assays also demonstrated that the intact *WDFY4* augmented MDA5-induced NF- κ B signal and apoptosis, but to lesser extent than tr-*WDFY4*. The differences in function between the intact *WDFY4* and tr-*WDFY4* should be further examined, but the lack of predicted *WDFY4* protein domains (BEACH and WD domains) may have a marked impact on tr-*WDFY4* function. Moreover, the suppression of IRF3 activity observed in the reporter assays suggests the role of *WDFY4* in interferon signaling pathway. Because *WDFY4* was shown to be downregulated in SLE patients⁴⁵, *WDFY4*

isoforms may control the balance between interferon and NF-κB pathways, which should be further examined.

The lack of an association for rs7919656 (sQTL variant) in the European patients might be due to a lack of statistical power, because it diminished to 0.32 if a moderate effect size (OR=1.5) was assumed. However, as rs11101462, which displayed a positive association signal in Europeans, had an eQTL effect for *WDFY4* in peripheral blood samples with higher expression in the risk allele (supplementary table S2), *WDFY4* may also have a role in CADM in Europeans. The genetic heterogeneity observed between these populations might be partially explained by environmental factors. Because the extent of MDA5 signal transduction depends on the characteristic of ligands (viruses) such as their length³⁰, the effects of *WDFY4* isoforms on the MDA5 signaling pathway may also depend on the ligands. Therefore, if viruses trigger the disease and the type of viruses is different between the populations, the genetic role of the two variants and their related *WDFY4* isoforms may also differ. The higher incidence of RPIP reported in Asian populations^{4,46} may indeed suggest etiological heterogeneity in CADM.

An autoimmune reaction to MDA5 may be a central event in CADM pathogenesis, because CADM is characterized by the appearance of anti-MDA5-Abs. This phenomenon was also observed in 72.2% (n=13/18) of the CADM patients evaluated, and these anti-MDA5-positive sera from patients could co-immunoprecipitate tr-*WDFY4* proteins. Interestingly, the serum from an anti-MDA5-negative patient could co-immunoprecipitate tr-*WDFY4* proteins, which might suggest the presence of anti-*WDFY4* antibody. An inflammatory condition following exposure to antigens derived from apoptotic cells is one of the main mechanisms that trigger autoimmune disorders as shown in the animal models.⁴⁷ Therefore, our findings that tr-*WDFY4*

enhanced both inflammatory signals and apoptosis induced by MDA5 suggest that tr-WDFY4 has a critical role in the autoimmune response to MDA5 and possibly to WDFY4.

Moreover, because tr-WDFY4 augmented the NF- κ B signals induced by PRRs, tr-WDFY4 may be also involved in the pathogenesis of RPIP. However, the high mortality rate of RPIP might have precluded the CADM patients with RPIP from participating in the study. Therefore, our samples might have underestimated the role of *WDFY4* variant in this disease subset, particularly in patients with RPIP. To determine whether the *WDFY4* variant can be used as a predictive biomarker for RPIP, a prospective study of new-onset CADM patients (or anti-MDA5-positive patients) is needed. Meanwhile, by assessing the environmental exposure, viral infections in particular, the role of gene-environmental interactions can be examined. As RPIP in CADM is still refractory to intense immunosuppressive therapies, tr-WDFY4 would be a candidate target in the treatment of CADM.

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Contributors

Y.Kochi designed the study and drafted the manuscript. T.S., H.K., and Y.K. directed the Research Team for Autoimmune Diseases and revised the manuscript. Y.Momozawa and M.K. performed the genotyping for GWAS. Y.Kochi, Y.Kamatani, and A.Takahashi analyzed the GWAS data. Y.Kochi, E.K., and K.I. performed eQTL analysis and wPGSA. Y.Kochi and A.S. conducted the reporter assays and immunoprecipitation. K.M. performed apoptosis assays and immunofluorescence staining. R.H., K.O., S.T., and T.M. assayed anti-MDA5 Abs and performed immunoprecipitation analysis using the sera from CADM patients. The following authors contributed to the Research Team for Autoimmune Diseases and its sample collection: Y.Kondo, M.F., M.J., Y.T., T.K.,

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Conflict of interest

The authors have declared that no conflict of interest exists.

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Table 1 Association analysis of rs7919656 with IIM

Disease	Numbers		Allele A frequency		Allelic comparison		
	Patients	Controls	Patients	Controls	OR (95% CI)	χ^2	P value
IIM total	576	6270	0.163	0.129	1.32 (1.12-1.56)	10.9	0.00095
IIM without CADM	543	6270	0.151	0.129	1.20 (1.01-1.43)	4.3	0.037
PM	236	6270	0.157	0.129	1.26 (0.98-1.62)	3.2	0.076
DM total	340	6270	0.168	0.129	1.36 (1.11-1.68)	8.6	0.0034
DM without CADM	307	6270	0.147	0.129	1.16 (0.92-1.46)	1.6	0.20
CADM	33	6270	0.364	0.129	3.87 (2.23-6.55)	32.0	1.5 x 10 ⁻⁸

IIM, idiopathic inflammatory myopathy; CADM, clinically amyopathic dermatomyositis;

DM, dermatomyositis; PM, polymyositis; OR, odds ratio; CI, confidence interval.

Figure legends

Figure 1 Association of the *WDFY4* locus with clinically amyopathic dermatomyositis (CADM) and its splicing QTL effect on a truncated *WDFY4* isoform. (A) Regional association plot of the *WDFY4* locus with CADM in Japanese drawn by LocusZoom. The recombination rate estimate is based on East Asian genotype data from the 1000 Genomes project (hg19 coordinates). The dot line indicates the genome-wide significance level ($P = 5 \times 10^{-8}$). (B) eQTL analysis of the *WDFY4* gene and its new exon. RPKM, reads per kilobase per million mapped reads. The line in the middle of the box is the median, and the box edges are the 25th and 75th percentiles of 373 samples. (C) Splicing isoform of *WDFY4*. The RNA-seq data from two Guevadis project samples representing genotypes of rs7919656 were displayed using the Integrative Genomics Viewer (IGV) (top). The new exon extends the exon 36 sequence and introduces a new stop codon (TGA in green). The genomic structure of the reference *WDFY4* transcript is shown (bottom). (D) The protein structures of the *WDFY4* isoforms. The reference *WDFY4* contains a BEACH domain and five WD domains, which are lacking in tr-*WDFY4*. The 11 a.a. sequences at C-terminal of tr-*WDFY4* is translated by the new exon. (E) Western blot analysis of V5-tagged, *WDFY4* isoforms that were transfected into HEK-293 cells.

Figure 2 Trans-eQTL analysis of the *WDFY4* variant and its application to gene set analysis. **(A)** Examples of the trans-eQTL effect of the *WDFY4* variant (rs7919656). **(B)** Application of the results of trans-eQTL analysis to wPGSA. We evaluated trans-eQTL effects (β) of *WDFY4* variant (rs7919656) for distal genes (Gene 1~N) by using wPGSA and estimated the transcriptional factors (TF1, TF2...) responsible for the regulation. **(C)** Transcriptional regulatory network of TFs responsible for the trans-eQTL effects of *WDFY4*. The nodes represent the significant TFs identified by wPGSA (FDR < 0.05). The circle node represents positive regulation for the trans-eQTL effects and the box node represents negative regulation. Those nodes regulating many other TFs were placed in a higher position and those regulated by many other TFs were placed to the right. The node colors and their density indicate the trans-eQTL effect (β) of *WDFY4* variant for the TF itself (red for positive β and blue for negative β). The width of the directed line represents the probability of transcriptional regulation of a TF over other TF gene. **(D)** NF- κ B activity was evaluated using reporter assays in HEK-293 cells that were transfected with *WDFY4* isoforms and were stimulated or not with TNF- α (1ng/ml for 2 hours). Means and s.d. are shown for six technical replicates. The experiment was independently repeated three times, and the representative data were shown. * P < 0.05 by ANOVA with post-hoc Tukey's test.

Figure 3. WDFY4 isoforms interact with pattern recognition receptors (PRR) and augment their signals. (A) Each transfected PRR signal was evaluated by NF- κ B reporter assays of HEK-293 cells co-transfected with each *WDFY4* isoform. *UNC93B1* was co-transfected in all the experiments for TLR7 and TLR9. Black bars indicate the stimulation reagents used (Poly (I:C), 10 μ g/ml; LPS, 1 μ g/ml; Imiquimod, 1 μ g/ml; ODN2006, 10 μ g/ml; high molecular weight poly (I:C), 1 μ g/ml). Means and s.d. are shown for four technical replicates. Each experiment was independently repeated three times, and the representative data were shown. * $P < 0.05$ by ANOVA with post-hoc Tukey's test. (B) Immunoprecipitation and western blotting analysis of the HEK-293 cells. Each PRR was immunoprecipitated with anti-FLAG Ab. Co-immunoprecipitated WDFY4 isoforms were then detected by western blotting (WB) using anti-V5 Ab. Each experiment was repeated twice, and the representative data were shown.

Figure 4 Functional analysis of WDFY4 isoforms as evaluated by apoptosis assays and IRF3 reporter assays. **(A)** Expression of MDA5 was upregulated by stimulation of HepG2 cells with HMW poly (I:C) for 24 h. The MDA5 transcript was quantified by TaqMan PCR using GAPDH expression as an internal control. **(B)** tr-WDFY4 enhanced apoptosis in HepG2 cells. Apoptotic cells were determined by assay of caspase activity using flow cytometry. Means and s.d. are shown for four technical replicates. $*P < 0.05$ by ANOVA with post-hoc Tukey's test. **(C)** Induction of Type I interferon in HEK-293 cells that were transfected with WDFY4 isoforms and MDA5 (or TLR3) was evaluated using IRF3 reporter assays. Black bars indicate the stimulation reagents used (Poly (I:C), 10 μ g/ml; high molecular weight poly (I:C), 1 μ g/ml). Means and s.d. are shown for four technical replicates. $*P < 0.05$ by ANOVA with post-hoc Tukey's test.

Figure 5 Interaction of MDA5 and tr-WDFY4. (A) Cellular localization of transfected V5-tagged WDFY4 isoforms and FLAG-tagged MDA5 was evaluated by immunofluorescence staining DAPI was used for counter staining of nuclei. Scale bars: 10µm. 100X objective lens was used. (B) Immunoprecipitation (IP) analysis using sera from CADM patients. MDA5 and tr-WDFY4 were co-transfected into HEK-293 cells, and the cell lysates were immunoprecipitated with sera from CADM patients (three anti-MDA5 positive patients and an anti-MDA5 negative patient. For comparison to another subset of myositis, sera from an anti-aminoacyl tRNA synthetase (ARS) positive DM patient as well as a healthy control (HC) were evaluated. Immunoprecipitated MDA5 proteins were detected by western blotting (WB) with anti-FLAG Ab (upper panel), and co-immunoprecipitated tr-WDFY4 proteins were detected with anti-His Ab (lower panel).

(Splicing variant of WDFY4 augments MDA5 signaling and the risk of clinically amyopathic dermatomyositis

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ABSTRACT

Objectives Idiopathic inflammatory myopathies (IIM) are a heterogeneous group of rare autoimmune diseases in which both genetic and environmental factors play important roles. To identify genetic factors of IIM including polymyositis (PM), dermatomyositis (DM), and clinically amyopathic DM (CADM), we performed the first genome-wide association study (GWAS) for IIM in an Asian population.

Methods We genotyped and tested 496,819 SNPs for association using 576 IIM patients and 6,270 control subjects. We also examined the causal mechanism of disease associated variants by *in silico* analyses using publicly available data sets as well as by *in vitro* analyses using reporter assays and apoptosis assays.

Results We identified a variant in *WDFY4* that was significantly associated with CADM (rs7919656; odds ratio (OR)=3.87; $P=1.5\times10^{-8}$). This variant had a cis-splicing quantitative trait locus (QTL) effect for a truncated *WDFY4* isoform (tr-*WDFY4*), with higher expression in the risk allele. Trans-expression QTL (eQTL) analysis of this variant showed a positive correlation with the expression of NF- κ B associated genes. Furthermore, we demonstrated that both *WDFY4* and tr-*WDFY4* interacted with pattern recognition receptors such as TLR3, TLR4, TLR9, and MDA5 and augmented the NF- κ B activation by these receptors. *WDFY4* isoforms also enhanced MDA5-induced apoptosis, to a greater extent in the tr-*WDFY4*-transfected cells.

Conclusions As CADM is characterized by the appearance of anti-MDA5 autoantibodies and severe lung inflammation, the *WDFY4* variant may play a critical role in the pathogenesis of CADM.

INTRODUCTION

Idiopathic inflammatory myopathies (IIM) are autoimmune conditions characterized by muscle weakness and inflammation, and the most common types are polymyositis (PM) and dermatomyositis (DM).^{1,2} Clinically amyopathic DM (CADM), a subset of DM, lacks distinct muscle features but often manifests rapidly progressive interstitial pneumonia (RPIP).³⁻⁶ The mortality rate of CADM at 6 months from diagnosis reaches approximately 50%, making it one of the most fatal autoimmune diseases.^{7,8} The appearance of autoantibodies such as anti-MDA5-antibodies in CADM suggests a role for autoimmunity in the pathogenesis.⁹⁻¹¹ Although the etiology of IIM remains unknown, virus infection may be the major environmental factor.¹² In addition, IIM patients and their close relatives are more likely to develop other autoimmune diseases, suggesting shared genetic factors.¹³⁻¹⁵ In fact, a previous genome-wide association studies (GWAS) and candidate gene analyses in European populations demonstrated multiple loci that were shared with other autoimmune diseases.¹⁵⁻¹⁸

However, when compared with other autoimmune diseases, little is known about the genetic background of IIM. This may be due to the rarity of disease, precluding researchers from designing sufficiently powered studies. In addition, because IIMs are a heterogeneous group of rare disorders, an overall pooled analysis might also reduce the power for detecting loci specific to certain subtypes. Therefore, as suggested¹⁹, studies of disease subsets are needed to increase the power for detecting subset-specific genetic components. Here, to elucidate genetic loci associated with IIM, we performed a GWAS for IIM in the Japanese population. This study was the first GWAS for IIM in an Asian population. We also performed a GWAS for CADM as a subset analysis, which was the first such study in the world.

Methods

Subjects

We enrolled 592 IIM cases (mean age, 55.5±14.2 years; female, 74.6%) from 18 medical institutes in Japan. PM/DM fulfilled the definite or probable diagnostic criteria of Bohan and Peter.¹ The diagnosis of CADM was based on the criteria by Sontheimer et al.^{3,6} We enrolled 6270 control subjects without history of autoimmune diseases (mean age, 57.0±13.8 years; female, 48.2%) through the Biobank project²⁰. The European subjects (21 CADM cases) were recruited as part of an international myositis genetics consortium (MYOGEN),¹⁷ and four controls for each case (84 controls) were matched for ethnicity using principal components analysis (PCA) coordinates.²¹ All subjects were provided with written informed consent for participation in the study as approved by the ethical committee of each institutional review board.

GWAS

We genotyped case samples using HumanOmniExpressExome BeadChip and control samples using HumanOmniExpress BeadChip and HumanExome BeadChip (Illumina). For quality control, we excluded single nucleotide polymorphisms (SNPs) with a call rate <0.99, a mean allele frequency (MAF) <0.05, and those not in Hardy-Weinberg equilibrium in controls ($P<1\times10^{-6}$). We removed samples that showed second-degree relatedness or closer by an identity-by-state method. We performed PCA using four reference populations from HapMap data including CEU, YRI, JPT and CHB using SmartPCA software.²² We identified outliers not belonging to the JPT/CHB cluster by using the top two principal components. After this screening, we selected 576 cases and 6270 controls for subsequent analysis. This sample size had statistical power >0.5 for

detecting common variants with moderate effects (MAF=0.5 and OR=1.5) as calculated by QUANTO.²³ The European samples were genotyped by the Immunochip (Illumina)¹⁸. We performed genotype imputation within the *WDFY4* locus by Minimac²⁴ using the 1000 Genomes Project Asian data as reference.

eQTL analysis

Both cis- and trans- eQTL analyses were performed using Matrix eQTL with the linear model.²⁵ We used the Geuvadis project data²⁶ of European samples (n=373), “GD462.GeneQuantRPKM.50FN.samplename.resk10.norm.txt” for the gene level eQTL analysis and “GD462.ExonQuantCount.45N.50FN.samplename.resk10.txt” for the exon level eQTL analysis.

wPGSA

To predict the activities of transcriptional regulators responsible for observed gene expression changes resulting from trans-eQTL effects of tr-*WDFY4*, we used weighted parametric gene set analysis (wPGSA)²⁷ with some modifications (supplementary figure S7). ChIP-seq data of multiple types of human cells were obtained from the GEO-database. In total, 3082 ChIP-seq data of 431 TFs were used. We used the trans-eQTL effects of *WDFY4* variant (rs7919656) on every gene (β obtained by Matrix-eQTL) as input, instead of the log-fold change of the gene expression.

Reporter assay

The coding sequences of *WDFY4* and tr-*WDFY4* were cloned into the pcDNA3.1D/V5-His-TOPO vector using cDNAs from EBV-immortalized B cells.

Human pattern recognition receptors were cloned into the pEF-BOS-FLAG vector (TLR3 was provided by Dr. Matsumoto, Hokkaido University; TLR4 and TLR9 were provided by Dr. Miyake, the University of Tokyo). UNC93B1 was cloned into the pcDNA5/FRT/V5-His vector. UNC93B1 was co-transfected for the experiments of TLR7 or TLR9, because it is essential to deliver these receptors to endolysosomes.²⁸ We cultured HEK-293 cells in DMEM supplemented with 10% fetal bovine serum. We transfected *WDFY4* expression vectors, the pGL4.32 vector (NF- κ B-response element and luc2P) and the pGL4.74 vector (hRluc/TK) (Promega) into cells using the X-tremeGENE HP Transfection Reagent (Roche). The p55C1B-Luc vector²⁹ (provided by Dr. Fujita, Kyoto University) was used instead of pGL4.32 for IRF3 reporter assays. After 18h incubation, the cells were stimulated with poly (I:C) (Enzo Life Sciences), LPS (Sigma-Aldrich), imiquimod (InvivoGen), ODN2006 (InvivoGen), or high molecular weight poly (I:C)³⁰ (provided by Dr. Fujita) for 4h. We then collected the cells and measured luciferase activity using Dual-Luciferase Reporter Assays according to the manufacturer's protocol (Promega).

Immunoprecipitation and western blotting

HEK-293 cells were transfected with each *WDFY4* isoform and PRR and cultured for 24h. The cells were lysed in RIPA buffer³¹ and then incubated with Dynabeads protein-G (Life Technologies) coated with anti-FLAG-antibody (Sigma-Aldrich) and incubated at 4°C for 30min. The beads were then washed and boiled in SDS buffer before electrophoresis through NuPAGE Tris-Acetate gels. The proteins were transferred to PDVF membranes (Millipore), which were then probed with anti-V5-antibody (Invitrogen), visualized using ECL reagent (GE Healthcare), and

detected using LAS-3000 (Fuji Film). We also performed immunoprecipitation using the sera from patients, instead of anti-FLAG-antibody.

Apoptosis assay

HepG2 Cells were transfected with each *WDFY4* isoform using the Lipofectamine 3000 reagent (Invitrogen). The same vector with cloned *LacZ* was also transfected as a mock. Cells were incubated for 24h and stimulated with 5 μ g of poly (I:C). The cells were permeabilized and stained with CaspACE FITC-VAD-FMK (Promega) and anti-His-Alexa 647. Caspase activity was evaluated in the His-positive cells using the BD Accuri C6 flow cytometer (BD Biosciences) to detect apoptotic cells.

Immunofluorescence staining

HEK-293 cells were grown on coverslips and transfected with the vectors for *WDFY4* isoforms and *MDA5*. Cells were fixed in 4% paraformaldehyde, permeabilized in 0.25% Triton X-100, and stained with anti-V5 and anti-mouse IgG-Alexa488 (Invitrogen) for *WDFY4* and anti-FLAG-Cy3 (Sigma-Aldrich) for *MDA5* detection. DAPI was used for counter-staining. Images were acquired with the confocal microscope N-SIM (Nikon) using 100X objective lens. The data were analyzed in 3D-SIM mode by using NIS-Elements software (Nikon).

Measurement of anti-MDA5

We measured anti-MDA5 autoantibodies in the sera from the CADM patients (18 sera were available) by protein-immunoprecipitation.⁹

Statistics

We tested the association of SNPs by allelic test using Plink 1.9,³² setting the genome-wide significance threshold at $\alpha=5\times10^{-8}$. We used a quantile-quantile plot to evaluate the potential effects of population stratification. We used analysis of variance (ANOVA) with post-hoc Tukey’s test in the reporter assays and apoptosis assays.

RESULTS

GWAS for IIM

We genotyped the Japanese samples using Illumina SNP arrays for over 960,000 SNPs. After quality control filtering, the effective sample size was 576 IIM cases and 6,270 controls, and 496,819 SNPs were tested. The genomic inflation factor λ_{GC} was 1.024 (supplementary figure S1), suggesting a negligible effect of population stratification. Cases comprised 236 PM and 340 DM patients including 33 CADM patients, and GWASs for these disease subsets were also performed. No significant association signal ($P < 5 \times 10^{-8}$) was found in any of total IIM, PM or DM (supplementary figure S2). However, when CADM patients were evaluated alone, a significant association was observed for an intronic SNP of *WDFY4* (WDFY Family Member 4; rs7919656; OR=3.87; 95% CI=2.23–6.55; $P=1.5 \times 10^{-8}$) (figure 1A and table 1). This SNP showed a nominal association with IIM, even when CADM patients were excluded ($P=0.037$). In European CADM patients, although no obvious association signal was observed in rs7919656, distinct association signals ($r^2 < 0.2$ with rs7919656) were observed for the neighboring variants (rs11101462, $P=0.0092$; rs2889697, $P=0.0058$, supplementary figure S3)

Splicing QTL effect of WDFY4 variant on truncated isoform

To unravel the causal mechanism of the *WDFY4* locus in this disease subset, we first searched for candidate functional variants. Among the variants in strong linkage disequilibrium (LD) with rs7919656 ($r^2 > 0.8$) in the 1000 genome project Asian samples, there is no coding variant that altered the amino-acid sequences of neighboring genes (supplementary figure S4). We next searched the expression quantitative trait locus

(eQTL) data of lymphoblastoid B-cells provided by the Geuvadis project.²⁶ When correlations of the SNP (rs7919656) genotype and expression of both the gene and exons were evaluated, a significant eQTL effect for an alternative exon of *WDFY4* was found (figure 1B). As no obvious eQTL effect was observed for expression of the whole gene or of neighboring exons (supplementary figure S5) this variant or variants in LD may have a splicing QTL effect for this *WDFY4* isoform. Among such variants, two SNPs in intron 35 (rs10776647/rs10776648; figure 1C and supplementary figure S4) were candidate causal variants in this region; however, they did not disrupt classical splicing associated motifs. The alternative exon extends exon 36 of *WDFY4*, which introduces an alternate stop codon and truncates the protein (figure 1C, D). We verified the existence of this truncated isoform (tr-*WDFY4*) by cloning it with cDNA from EBV-immortalized B cells and expressing it in HEK-293 cells (figure 1E).

Trans eQTL effect of *WDFY4* variant on NF-κB activity

WDFY4 encodes WDFY family member 4, which has a predicted BEACH domain and five WD domains at the C-terminal side³³ (figure 1D). Although *WDFY4* is expressed in dendritic cells, neutrophils, B-cells and macrophages (supplementary figure S6), to our knowledge, there have been no studies into its protein function. To obtain insight regarding the function of *WDFY4*, we performed trans-eQTL analysis of the variant using the same expression data of lymphoblastoid B-cells. A total of 387 genes were correlated with the number of risk alleles (uncorrected $P<0.05$), although no gene displayed genome-wide significant correlation (FDR<0.05). Notably, 279 genes (72.1%) were positively correlated, indicating that upregulated expression of tr-*WDFY4* results in upregulation of these genes. Among the top 10 genes with the largest

trans-eQTL effects (β as calculated in the Matrix eQTL analysis), *TYK2* and *PTPN6* are well known regulators of signal pathways in B-cells (figure 2A). We then sought to identify the transcriptional factors (TFs) present downstream of tr-*WDFY4* by weighted parametric gene set analysis (wPGSA).²⁷ wPGSA utilizes all available ChIP-seq data obtained from multiple types of human cells and predicts the activities of transcriptional regulators responsible for observed gene expression changes (figure 2B and supplementary figure S7). When trans-eQTL effects (β) were used as input, among the 431 TFs evaluated, 6 TFs displayed significant associations (FDR<0.05) (figure 2C and supplementary table S1). We focused on TF65 (*RELA*/p65), because it comprises nuclear factor-kappa B (NF- κ B) heterodimers that are one of the most important TFs in the immune system.³⁴ Based on these findings, we assumed that the NF- κ B pathway is activated downstream of WDFY4. Indeed, NF- κ B reporter assays showed that both WDFY4 and tr-WDFY4 enhanced NF- κ B activity when transfected into HEK-293 cells with stimulation of TNF- α (figure 2D).

WDFY4 augments signals of innate pattern recognition receptors

These observations, as well as a recent report that WDFY1, another member of the WDFY gene family, augmented TLR3 and TLR9 signaling³⁵, motivated us to investigate the role of WDFY4 in PRR signaling. We chose TLR3/TLR4/TLR7/TLR9 and MDA5 for analysis considering their implied roles in disease pathogenesis^{4 36 37}, as well as their potential to activate NF- κ B.³⁸ We first performed NF- κ B reporter assays by simultaneously transfecting each WDFY4 isoform with each PRR. While both WDFY4 isoforms enhanced NF- κ B signaling of TLR3/TLR4/TLR9 and MDA5, they had a negligible effect on TLR7 signaling (figure 3A). Of note, tr-WDFY4 markedly

enhanced the MDA5 signal when stimulated with poly (I:C), a synthetic analog of double-stranded RNA. We also examined the impact of WDFY4 isoforms on cytokine production by quantifying the levels of IL-8 in the cell culture supernatants. Although both isoforms significantly increased the IL-8 production in steady state, the difference to mock transfectant became insignificant when cells were stimulated with poly (I:C) (supplementary figure S8). Next, we examined the physical interaction of WDFY4 isoforms and these PRRs by co-immunoprecipitation. Both V5-tagged WDFY4 isoforms interacted with these FLAG-tagged PRRs to greater or lesser degrees (figure 3B). The strongest interaction was observed between tr-WDFY4 and MDA5, which was consistent with the findings of reporter assays.

MDA5-mediated sensing of virus triggers not only endogenous immune responses but also cellular apoptosis to eliminate virus-infected cells.³⁹ Therefore, to further evaluate the function of WDFY4 in the context of MDA5 signaling, we performed apoptosis assays. We used HepG2 cells for these assays, because stimulation of these cells with poly (I:C) is accompanied by upregulation of MDA5 and results in the induction of apoptosis⁴⁰ (figure 4A). When HepG2 cells were transfected with the WDFY4 isoforms and stimulated with/without poly (I:C), apoptosis was augmented to a greater extent in the tr-WDFY4-transfected cells than in the WDFY4-transfected cells (figure 4B). Again, this result is consistent with the observations regarding these isoforms in the NF- κ B assays. As the apoptosis induced by MDA5 was reported to be independent of type I interferon signals⁴¹, we evaluated the effect of WDFY4 isoforms on type I interferon induction by co-transfected MDA5 using IRF3 reporter assays²⁹. For comparison, we also examined TLR3 signaling. While no effect of WDFY4 isoforms on TLR3 induction of interferon was detected, MDA5 induction of interferon was markedly

repressed when the WDFY4 isoforms were co-transfected with MDA5 (figure 4C).

These findings suggested that the WDFY4 isoforms are able to interact with MDA5 and alter its signaling in cells. This possibility was further supported by the observations that transfected WDFY4 isoforms and MDA5 co-localized in the cytoplasm of cells as assessed using immunofluorescence (figure 5A), and that the anti-MDA5-positive sera from patients could co-immunoprecipitate tr-WDFY4 proteins (figure 5B).

DISCUSSION

We here demonstrated a significant association between a variant in *WDFY4* and CADM susceptibility. Association of *WDFY4* locus has been previously reported with systemic lupus erythematosus (SLE)⁴² and rheumatoid arthritis.⁴³ A recent study reported that the SLE risk variant (rs2663052) also had a splicing QTL effect on *WDFY4*.⁴⁴ However, rs2663052 is not in tight LD with the CADM risk variant (rs7919656, $r^2=0.46$ in the Asian samples), and rs2663052 displayed a weaker association signal in CADM in the present study ($P=0.0012$). A missense variant (rs7097397), which is in moderate LD with rs7919656 ($r^2=0.58$ in the Asian samples), may be another candidate causal variant in SLE⁴², but the association in CADM was moderate ($P=0.0022$).

The fact that the CADM risk variant was also associated with upregulated expression of tr-*WDFY4* suggested that *WDFY4* was a strong candidate gene in this locus. Although the biological function of *WDFY4* was unknown, our analysis by wPGSA utilizing the trans-eQTL effects of *WDFY4* variant implied that NF- κ B was involved in the downstream regulation of *WDFY4*. Indeed, as demonstrated by *in vitro* assays, tr-*WDFY4* augmented the NF- κ B signals induced by PRRs including MDA5. Our *in vitro* assays also demonstrated that the intact *WDFY4* augmented MDA5-induced NF- κ B signal and apoptosis, but to lesser extent than tr-*WDFY4*. The differences in function between the intact *WDFY4* and tr-*WDFY4* should be further examined, but the lack of predicted *WDFY4* protein domains (BEACH and WD domains) may have a marked impact on tr-*WDFY4* function. Moreover, the suppression of IRF3 activity observed in the reporter assays suggests the role of *WDFY4* in interferon signaling pathway. Because *WDFY4* was shown to be downregulated in SLE patients⁴⁵, *WDFY4*

isoforms may control the balance between interferon and NF- κ B pathways, which should be further examined.

The lack of an association for rs7919656 (sQTL variant) in the European patients might be due to a lack of statistical power, because it diminished to 0.32 if a moderate effect size (OR=1.5) was assumed. However, as rs11101462, which displayed a positive association signal in Europeans, had an eQTL effect for *WDFY4* in peripheral blood samples with higher expression in the risk allele (supplementary table S2), *WDFY4* may also have a role in CADM in Europeans. The genetic heterogeneity observed between these populations might be partially explained by environmental factors. Because the extent of MDA5 signal transduction depends on the characteristic of ligands (viruses) such as their length³⁰, the effects of *WDFY4* isoforms on the MDA5 signaling pathway may also depend on the ligands. Therefore, if viruses trigger the disease and the type of viruses is different between the populations, the genetic role of the two variants and their related *WDFY4* isoforms may also differ. The higher incidence of RPIP reported in Asian populations^{4,46} may indeed suggest etiological heterogeneity in CADM.

An autoimmune reaction to MDA5 may be a central event in CADM pathogenesis, because CADM is characterized by the appearance of anti-MDA5-Abs. This phenomenon was also observed in 72.2% (n=13/18) of the CADM patients evaluated, and these anti-MDA5-positive sera from patients could co-immunoprecipitate tr-*WDFY4* proteins. Interestingly, the serum from an anti-MDA5-negative patient could co-immunoprecipitate tr-*WDFY4* proteins, which might suggest the presence of anti-*WDFY4* antibody. An inflammatory condition following exposure to antigens derived from apoptotic cells is one of the main mechanisms that trigger autoimmune disorders as shown in the animal models.⁴⁷ Therefore, our findings that tr-*WDFY4*

enhanced both inflammatory signals and apoptosis induced by MDA5 suggest that tr-WDFY4 has a critical role in the autoimmune response to MDA5 and possibly to WDFY4.

Moreover, because tr-WDFY4 augmented the NF- κ B signals induced by PRRs, tr-WDFY4 may be also involved in the pathogenesis of RPIP. However, the high mortality rate of RPIP might have precluded the CADM patients with RPIP from participating in the study. Therefore, our samples might have underestimated the role of *WDFY4* variant in this disease subset, particularly in patients with RPIP. To determine whether the *WDFY4* variant can be used as a predictive biomarker for RPIP, a prospective study of new-onset CADM patients (or anti-MDA5-positive patients) is needed. Meanwhile, by assessing the environmental exposure, viral infections in particular, the role of gene-environmental interactions can be examined. As RPIP in CADM is still refractory to intense immunosuppressive therapies, tr-WDFY4 would be a candidate target in the treatment of CADM.

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Contributors

Y.Kochi designed the study and drafted the manuscript. T.S., H.K., and Y.K. directed the Research Team for Autoimmune Diseases and revised the manuscript. Y.Momozawa and M.K. performed the genotyping for GWAS. Y.Kochi, Y.Kamatani, and A.Takahashi analyzed the GWAS data. Y.Kochi, E.K., and K.I. performed eQTL analysis and wPGSA. Y.Kochi and A.S. conducted the reporter assays and immunoprecipitation. K.M. performed apoptosis assays and immunofluorescence staining. R.H., K.O., S.T., and T.M. assayed anti-MDA5 Abs and performed immunoprecipitation analysis using the sera from CADM patients. The following authors contributed to the Research Team for Autoimmune Diseases and its sample collection: Y.Kondo, M.F., M.J., Y.T., T.K.,

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M.K., H.Kohsaka, T.S., and K.Y. M.K. directed the analysis of the BioBank samples.
R.G.C., H.C., J.V., and H.M. collected the European samples. S.R. and J.A.L. analyzed
European CADM data.

Conflict of interest

The authors have declared that no conflict of interest exists.

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Table 1 Association analysis of rs7919656 with IIM

Disease	Numbers		Allele A frequency		Allelic comparison		
	Patients	Controls	Patients	Controls	OR (95% CI)	χ^2	P value
IIM total	576	6270	0.163	0.129	1.32 (1.12-1.56)	10.9	0.00095
IIM without CADM	543	6270	0.151	0.129	1.20 (1.01-1.43)	4.3	0.037
PM	236	6270	0.157	0.129	1.26 (0.98-1.62)	3.2	0.076
DM total	340	6270	0.168	0.129	1.36 (1.11-1.68)	8.6	0.0034
DM without CADM	307	6270	0.147	0.129	1.16 (0.92-1.46)	1.6	0.20
CADM	33	6270	0.364	0.129	3.87 (2.23-6.55)	32.0	1.5×10^{-8}

IIM, idiopathic inflammatory myopathy; CADM, clinically amyopathic dermatomyositis;

DM, dermatomyositis; PM, polymyositis; OR, odds ratio; CI, confidence interval.

Figure legends

Figure 1 Association of the *WDFY4* locus with clinically amyopathic dermatomyositis (CADM) and its splicing QTL effect on a truncated *WDFY4* isoform. (A) Regional association plot of the *WDFY4* locus with CADM in Japanese drawn by LocusZoom. The recombination rate estimate is based on East Asian genotype data from the 1000 Genomes project (hg19 coordinates). The dot line indicates the genome-wide significance level ($P=5\times10^{-8}$). (B) eQTL analysis of the *WDFY4* gene and its new exon. RPKM, reads per kilobase per million mapped reads. The line in the middle of the box is the median, and the box edges are the 25th and 75th percentiles of 373 samples. (C) Splicing isoform of *WDFY4*. The RNA-seq data from two Guevadis project samples representing genotypes of rs7919656 were displayed using the Integrative Genomics Viewer (IGV) (top). The new exon extends the exon 36 sequence and introduces a new stop codon (TGA in green). The genomic structure of the reference *WDFY4* transcript is shown (bottom). (D) The protein structures of the *WDFY4* isoforms. The reference WDFY4 contains a BEACH domain and five WD domains, which are lacking in tr-WDFY. The 11 a.a. sequences at C-terminal of tr-WDFY4 is translated by the new exon. (E) Western blot analysis of V5-tagged, WDFY4 isoforms that were transfected into HEK-293 cells.

Figure 2 Trans-eQTL analysis of the *WDFY4* variant and its application to gene set analysis. **(A)** Examples of the trans-eQTL effect of the *WDFY4* variant (rs7919656). **(B)** Application of the results of trans-eQTL analysis to wPGSA. We evaluated trans-eQTL effects (β) of *WDFY4* variant (rs7919656) for distal genes (Gene 1~N) by using wPGSA and estimated the transcriptional factors (TF1, TF2...) responsible for the regulation. **(C)** Transcriptional regulatory network of TFs responsible for the trans-eQTL effects of *WDFY4*. The nodes represent the significant TFs identified by wPGSA (FDR < 0.05). The circle node represents positive regulation for the trans-eQTL effects and the box node represents negative regulation. Those nodes regulating many other TFs were placed in a higher position and those regulated by many other TFs were placed to the right. The node colors and their density indicate the trans-eQTL effect (β) of *WDFY4* variant for the TF itself (red for positive β and blue for negative β). The width of the directed line represents the probability of transcriptional regulation of a TF over other TF gene. **(D)** NF- κ B activity was evaluated using reporter assays in HEK-293 cells that were transfected with *WDFY4* isoforms and were stimulated or not with TNF- α (1ng/ml for 2 hours). Means and s.d. are shown for six technical replicates. The experiment was independently repeated three times, and the representative data were shown. * P < 0.05 by ANOVA with post-hoc Tukey's test.

Figure 3. WDFY4 isoforms interact with pattern recognition receptors (PRR) and augment their signals. (A) Each transfected PRR signal was evaluated by NF- κ B reporter assays of HEK-293 cells co-transfected with each *WDFY4* isoform. *UNC93B1* was co-transfected in all the experiments for TLR7 and TLR9. Black bars indicate the stimulation reagents used (Poly (I:C), 10 μ g/ml; LPS, 1 μ g/ml; Imiquimod, 1 μ g/ml; ODN2006, 10 μ g/ml; high molecular weight poly (I:C), 1 μ g/ml). Means and s.d. are shown for four technical replicates. Each experiment was independently repeated three times, and the representative data were shown. * $P < 0.05$ by ANOVA with post-hoc Tukey's test. (B) Immunoprecipitation and western blotting analysis of the HEK-293 cells. Each PRR was immunoprecipitated with anti-FLAG Ab. Co-immunoprecipitated WDFY4 isoforms were then detected by western blotting (WB) using anti-V5 Ab. Each experiment was repeated twice, and the representative data were shown.

Figure 4 Functional analysis of WDFY4 isoforms as evaluated by apoptosis assays and IRF3 reporter assays. **(A)** Expression of MDA5 was upregulated by stimulation of HepG2 cells with HMW poly (I:C) for 24 h. The MDA5 transcript was quantified by TaqMan PCR using GAPDH expression as an internal control. **(B)** tr-WDFY4 enhanced apoptosis in HepG2 cells. Apoptotic cells were determined by assay of caspase activity using flow cytometry. Means and s.d. are shown for four technical replicates. $*P < 0.05$ by ANOVA with post-hoc Tukey's test. **(C)** Induction of Type I interferon in HEK-293 cells that were transfected with WDFY4 isoforms and MDA5 (or TLR3) was evaluated using IRF3 reporter assays. Black bars indicate the stimulation reagents used (Poly (I:C), 10 μ g/ml; high molecular weight poly (I:C), 1 μ g/ml). Means and s.d. are shown for four technical replicates. $*P < 0.05$ by ANOVA with post-hoc Tukey's test.

Figure 5 Interaction of MDA5 and tr-WDFY4. (A) Cellular localization of transfected V5-tagged WDFY4 isoforms and FLAG-tagged MDA5 was evaluated by immunofluorescence staining DAPI was used for counter staining of nuclei. Scale bars: 10µm. 100X objective lens was used. (B) Immunoprecipitation (IP) analysis using sera from CADM patients. MDA5 and tr-WDFY4 were co-transfected into HEK-293 cells, and the cell lysates were immunoprecipitated with sera from CADM patients (three anti-MDA5 positive patients and an anti-MDA5 negative patient. For comparison to another subset of myositis, sera from an anti-aminoacyl tRNA synthetase (ARS) positive DM patient as well as a healthy control (HC) were evaluated. Immunoprecipitated MDA5 proteins were detected by western blotting (WB) with anti-FLAG Ab (upper panel), and co-immunoprecipitated tr-WDFY4 proteins were detected with anti-His Ab (lower panel).

Fig. 1

Fig. 1

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Fig. 2

Fig. 2

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Fig. 3

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Fig. 5

Fig. 5

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Supplementary Table S1 TFs associated with the trans-eQTL effects of *WDFY4* variant

TF name	Gene name	Number of ChIP-seq data	t score	P value	Q value (FDR)
HSF1	<i>HSF1</i>	58	6.24	2.2E-10	9.7E-08
HJURP	<i>HJURP</i>	2	-13.42	2.7E-09	5.9E-07
TF65	<i>RELA</i>	66	5.16	1.2E-07	1.8E-05
RFX5	<i>RFX5</i>	7	3.55	0.00019	0.021
C2TA	<i>CIITA</i>	5	3.28	0.00053	0.042
VDR	<i>VDR</i>	16	3.25	0.00058	0.042

UniProt entry name was used for TFs, and HUGO nomenclature was used for genes.

Supplementary Table S2 Cis-eQTL effect of rs11101462 for *WDFY4*

SNP	Chr	Pos	Probe name	Probe position	SNP allele	Allele assessed	Z Score	P value	FDR
rs11101462	10	5E+07	6620022	5E+07	A/G	G	5.34	9.24E-08	1.61E-05

The data were extracted using the Blood eQTL browser (<http://genenetwork.nl/bloodeqtlbrowser/>).
The positions of SNP and probe were in the hg19 reference genome.

Supplementary Figure S1 Q-Q plot of GWAS for IIM in Japanese. All IIM, all idiopathic inflammatory myopathies; PM, polymyositis; DM, dermatomyositis; CADM, clinically amyopathic dermatomyositis. The observed (y axis) and the expected (x axis) P value is plotted for each SNP (dot), and the red line indicates the null hypothesis of no true association. Genomic inflation factor (λ_{GC}) was indicated. Deviation from the expected P -value distribution is evident only in the tail area in CADM, suggesting that population stratification was adequately controlled.

Supplementary Figure S2 Manhattan plots of GWAS for IIMs in Japanese.

All IIM, all idiopathic inflammatory myopathies; PM, polymyositis; DM, dermatomyositis; CADM, clinically amyopathic dermatomyositis

The image was created using the R package qqman. The red line indicates the genome-wide significance level ($P = 5 \times 10^{-8}$). Three SNPs (rs7919656, rs3747872 and rs3747874) satisfied the genome-wide significance level in CADM and were in strong LD with each other ($r^2 > 0.95$).

Supplementary Figure S3. Association of *WDFY4* locus with CADM in Europeans. Regional association plot was drawn by LocusZoom. The recombination rate estimate is based on European genotype data from the 1000 Genomes project (hg19 coordinates). The GWAS SNP for CADM in the Japanese (rs7919656) was colored in purple. The LD index (r^2) for each SNP with rs7919656 was also colored as presented in the left panel. The dot line indicates the significance level of $P = 5 \times 10^{-8}$ and $P = 0.05$.

Query SNP: rs7919656 and variants with r² >= 0.8

pos (hg19)	LD (r ²)	LD (D')	variant	Ref	Alt	AFR freq	AMR freq	ASN freq	EUR freq	SiPhy cons	Promoter histone marks	Enhancer histone marks	DNase	Proteins bound	eQTL tissues	Motifs changed	RefSeq genes	dbSNP func annot
chr10:50014917	0.85	0.96	rs2448540	T	C	0.59	0.44	0.81	0.37			6 organs				5 altered motifs	WDFY4	intronic
chr10:50015097	0.85	0.96	rs2457214	T	C	0.65	0.46	0.81	0.37			5 organs					WDFY4	intronic
chr10:50015211	0.84	0.95	rs2448539	T	G	0.64	0.54	0.81	0.51			5 organs	MUS			FXR	WDFY4	intronic
chr10:50019099	0.9	0.98	rs11101511	G	C	0.78	0.49	0.81	0.39			ESDR, SKIN, MUS				AP-1	WDFY4	intronic
chr10:50020323	0.89	0.98	rs2250389	T	C	0.92	0.59	0.81	0.53			SKIN, MUS				HNF4, RXRA	WDFY4	intronic
chr10:50024030	0.9	0.98	rs2620896	C	T	0.89	0.50	0.81	0.39							Cdc5	WDFY4	intronic
chr10:50026020	1	1	rs7919656	A	G	0.57	0.55	0.82	0.51							XBP-1	WDFY4	intronic
chr10:50029705	0.97	1	rs2620891	A	G	0.91	0.59	0.82	0.53			4 organs	BRST, SKIN				WDFY4	intronic
chr10:50030864	0.97	1	rs10776647	C	T	0.54	0.54	0.82	0.51							EBF, EWSR1, FLI1, TATA	WDFY4	intronic
chr10:50030932	0.97	1	rs10776648	A	G	0.57	0.55	0.82	0.51				BLD			Foxa, Nanog, SP1	WDFY4	intronic
chr10:50037720	0.97	1	rs2663051	G	C	0.91	0.59	0.82	0.53							Mrg, SZF1-1, Spz1	WDFY4	intronic
chr10:50037974	0.97	1	rs11101529	G	A	0.71	0.56	0.82	0.51							TCF4	WDFY4	intronic
chr10:50038285	0.95	1	rs11101530	G	A	0.50	0.43	0.81	0.36							AP-1, Pax-5	WDFY4	intronic
chr10:50038487	0.95	1	rs10857655	A	T	0.50	0.43	0.81	0.36							AP-1, HNF4	WDFY4	intronic
chr10:50038585	0.95	1	rs3747877	G	A	0.54	0.44	0.81	0.36							CDP, GR	WDFY4	intronic
chr10:50038720	0.95	1	rs3747875	G	T	0.36	0.41	0.81	0.36							Rad21, YY1	WDFY4	intronic
chr10:50038900	0.97	1	rs3747874	A	G	0.71	0.56	0.82	0.51							4 altered motifs	WDFY4	synonymous
chr10:50039253	0.95	1	rs7079766	G	T	0.50	0.43	0.81	0.36								WDFY4	intronic
chr10:50039528	0.95	1	rs7093474	C	T	0.50	0.43	0.81	0.36							Pou5f1	WDFY4	intronic
chr10:50040076	0.8	1	rs7908454	G	A	0.70	0.52	0.79	0.50								WDFY4	intronic
chr10:50040562	0.95	1	rs3747873	A	G	0.50	0.43	0.81	0.36							6 altered motifs	WDFY4	intronic
chr10:50040610	0.95	1	rs3747872	T	G	0.50	0.43	0.81	0.36				BLD			9 altered motifs	WDFY4	intronic
chr10:50041123	0.95	1	rs7900138	A	G	0.50	0.43	0.81	0.36							GATA, Mef2, SIX5	WDFY4	intronic
chr10:50041176	0.92	1	rs7913005	G	A	0.49	0.43	0.81	0.36							5 altered motifs	WDFY4	intronic
chr10:50041276	0.97	1	rs2620898	G	A	0.91	0.59	0.82	0.53							6 altered motifs	WDFY4	intronic
chr10:50041463	0.95	1	rs11101531	T	C	0.50	0.43	0.81	0.36							BCL, Brachyury, YY1	WDFY4	intronic
chr10:50041729	0.95	1	rs11101532	C	A	0.50	0.42	0.81	0.36							Ptfx2, Sox	WDFY4	intronic
chr10:50041734	0.97	1	rs2448545	C	G	0.94	0.59	0.82	0.53							EBF, Ptx2, TCF12	WDFY4	intronic
chr10:50042319	0.95	1	rs58214108	A	G	0.50	0.43	0.81	0.36			MUS				7 altered motifs	WDFY4	intronic
chr10:50042579	0.95	1	rs3860188	T	C	0.50	0.43	0.81	0.36			MUS				Esr2, Hdx	WDFY4	intronic
chr10:50042951	0.95	1	rs77819	A	G	0.54	0.44	0.81	0.36			PANC				4 altered motifs	WDFY4	intronic
chr10:50043141	0.95	1	rs877820	A	G	0.50	0.43	0.81	0.36			PANC				4 altered motifs	WDFY4	intronic
chr10:50043317	0.94	1	rs877821	G	C	0.17	0.39	0.81	0.36			PANC				18 altered motifs	WDFY4	intronic
chr10:50043766	0.94	1	rs3860189	A	G	0.50	0.43	0.81	0.36			ESDR				4 altered motifs	WDFY4	intronic
chr10:50043857	0.95	1	rs3860190	T	C	0.50	0.43	0.81	0.36			ESDR				Evi-1, Foxp1, SIX5	WDFY4	intronic
chr10:50043889	0.95	1	rs2002588	A	G	0.50	0.43	0.81	0.36			ESDR	BRN			Pou5f1	WDFY4	intronic
chr10:50043907	0.95	1	rs2002587	T	C	0.50	0.43	0.81	0.36			ESDR	BRN, BRN			8 altered motifs	WDFY4	intronic
chr10:50044824	0.95	1	rs6537580	G	A	0.50	0.43	0.81	0.36			MUS				GATA, Gfi1, Hoxa7	WDFY4	intronic
chr10:50045021	0.97	1	rs2620904	G	C	0.91	0.59	0.82	0.53			MUS				Foxl1, Foxo	WDFY4	intronic
chr10:50072102	0.91	0.96	rs2940727	G	A	0.91	0.59	0.82	0.53				BLD			4 altered motifs	WDFY4	intronic
chr10:50080311	0.88	0.98	rs1904596	A	G	0.96	0.63	0.81	0.59							8 altered motifs	WDFY4	intronic

Supplementary Figure S4 Candidate causal variants searched from the 1000 genome data by using HaploReg. All the variants in LD ($r^2 \geq 0.8$ in Asian samples) with the GWAS SNP (rs7919656) were shown. No missense variant was identified. HaploReg, <http://archive.broadinstitute.org/mammals/haploreg/haploreg.php>.

Supplementary Figure S5 Cis-eQTL analysis of WDFY4 variant (rs7919656) with the expression levels of exons using the Guevadis project samples (N= 373). The line in the middle of the box is the median, and the box edges are the 25th and 75th percentiles.

Supplementary Figure S6 Expression levels of *WDFY4* in immune cells and tissues. The expression data of *WDFY4* were extracted from the FANTOM5 database using ZENBU. The transcriptional start site (TSS) expression was quantified by cap analysis of gene expression (CAGE) and displayed as relative log expression (RLE). ZENBU, <http://fantom.gsc.riken.jp/zenbu/>.

Supplementary Figure S7 Application of the results of trans-eQTL analysis to wPGSA. Log-fold change (LFC) of expression data is usually evaluated in wPGSA. Instead, we evaluated trans-eQTL effects (β) of a variant for distal genes and identified the transcriptional factors responsible for the regulation.

Supplementary Figure S8 Impact of WDFY4 isoforms on cytokine production in HEK-293 cells. HEK-293 cells were transfected with WDFY4 isoforms and MDA5 with or without stimulation by high molecular weight (HMW) poly (I:C) at 1µg/ml for 24 hours. IL-8 in the cell culture supernatant was quantified by human IL-8 ELISA MAX kit (BioLegend) in a sandwich ELISA. Means and s.d. are shown for 6 technical replicates. **P* < 0.05 by ANOVA with post-hoc Tukey's test.