Table I *Continued*

Case[a]	Ethnic origin	Putative pathogenic mutation	Putative risk variant	Probable benign change	Copy-number alteration	Position of urethral opening[b]	Cryptorchidism	Micropenis
33	J		*HSD17B3* (p.G289S)	*HSD3B2* (p.R362W)		Anterior	No data	No data
34	J			*NR5A1* (g.IVS2-5G>A)		Posterior	No data	No data
35	J			*HOXB6* (p.P40S)		Posterior	No	Yes
36	J			***MAMLD1* (p.N675K)**		Posterior	No data	No data
37	J			*ESR2* (p.G67S)		Posterior	No	No
38	J			*EGF* (p.S16R) *BNC2* (p.I974V)		Middle	No data	No data

J, Japanese; V, Vietnamese.
Homozygous or hemizygous mutations/variations are boldfaced, and heterozygous substitutions are lightfaced.
[a]Cases 39–62 carried no nucleotide alterations in the target genes.
[b]Detailed clinical information was obtained only from 49 of the 62 patients.
[c]c.1995delTGAAGGCTATGAATGTCinsCAGAA; p.666delEGYECQinsRK.
[d]Homozygosity and heterozygosity of this mutation were described as a pathogenic defect and a disease-susceptible alteration, respectively.
[e]Copy-number gain of the region from Ypter to Yq11.223 and copy-number loss of the remaining Y chromosomal region.

Data (http://www.fruitfly.org/seq_tools/splice.html, 12 January 2015, date last accessed) (Reese *et al.*, 1997). Nucleotide deletions and insertions in the coding regions were assessed as 'probably damaging'.

Nucleotide alterations were classified into the following three groups: (i) putative pathogenic mutations: mutations that have been associated with 46,XY DSD or hitherto unreported nucleotide changes in causative genes that were assessed as 'probably damaging' or 'possibly damaging' by *in silico* analyses; (ii) putative risk variants: previously reported risk SNPs or novel substitutions in susceptibility genes, or rare SNPs in causative genes that were assessed as 'probably damaging' or 'possibly damaging'; and (iii) probable benign changes: nucleotide substitutions in causative/susceptible/candidate genes that were assessed as 'benign'. To determine the possible association between the SNPs (putative risk variants and probable benign changes) and disease risk, we compared allele frequencies in the patient group with those in the male general population. In the SNP analysis, we focused on Japanese patients, for whom the allele frequencies in the general population were available in the public database (dbSNP, http://www.ncbi.nlm.nih.gov/, 12 January 2015, date last accessed).

Statistical analysis

The statistical significance of the comparison of allele frequency in the patient group and the general population was evaluated using χ^2 and Fisher's exact probability tests.

Copy-number analyses

CNVs in the genome were screened by CGH using a catalog human array (8 × 60 k format, catalog number G4450A, Agilent Technologies), according to the manufacturers' instructions. In this study, we focused on copy-number alterations affecting genomic intervals larger than 1.5 Mb, which have a higher probability of being associated with disease phenotypes (Cooper *et al.*, 2011). We referred to the Database of Genomic Variants (http://projects.tcag.ca/variation/, 12 January 2015, date last accessed) to exclude known benign variants. Genomic structures of CNVs were characterized by FISH analysis.

Results

Identification and characterization of nucleotide substitutions

Eight putative pathogenic mutations were identified in seven patients (Table I and Fig. 1). The eight mutations consisted of three hemizygous missense mutations and one hemizygous deletion/insertion in *AR*, one heterozygous missense mutation in *HOXB6*, one heterozygous missense mutation in *BNC2*, and apparent homozygous mutations in *SRD5A2* and *HSD3B2*. Of these, the *AR* mutation in case 3 and the *SRD5A2* mutation in case 6 were previously identified in patients with 46,XY DSD (Melo *et al.*, 2003 in which the p.R841S mutation in *AR* was described as p.R840S; Sasaki *et al.*, 2003; van der Zanden *et al.*, 2012), while the other mutations were first identified in the present study.

Putative risk variants were identified in 30 patients (Table I and Supplementary Table SI). These variants included three known risk alleles for hypospadias and/or micropenis: rs2066476 in *HSD17B3*, rs2073043 in *MAMLD1* and rs9332964 in *SRD5A2* (Sasaki *et al.*, 2003; Fukami *et al.*, 2008; Sata *et al.*, 2010; Kalfa *et al.*, 2011; van der Zanden *et al.*, 2012). The SNPs in *HSD17B3* and *MAMLD1* were identified in the Japanese patient group and the male general population at similar frequencies. We also identified a rare SNP in the causative gene *CYP1A1* which was shared by the Japanese patients and the male

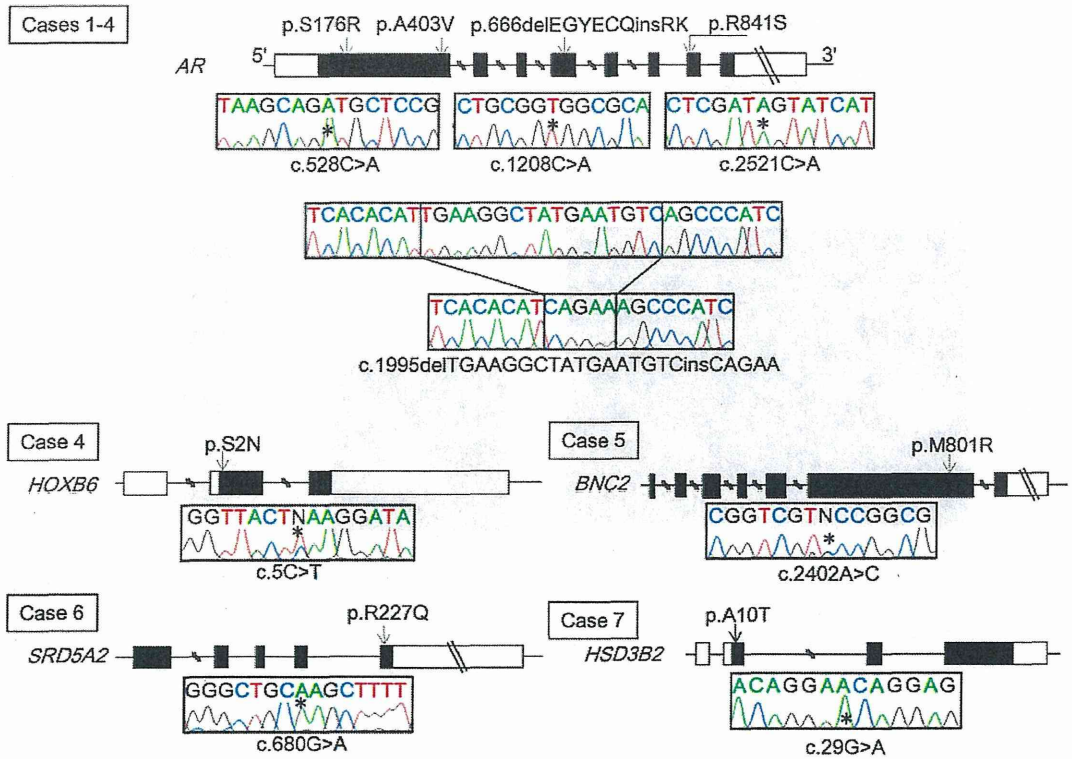

Figure 1 Putative pathogenic mutations identified in the present study. Genomic positions and chromatograms of the nucleotide substitutions are shown. Asterisks indicate the mutated nucleotides.

general population at a similar frequency, together with a SNP in *BMP4* whose frequency in the general population is unknown.

Probable benign changes were found in 13 patients (Table 1 and Supplementary Table S1). These substitutions included a rare SNP in *EGF* which was identified in the patient group and in the general population at similar frequency. We also detected SNPs in *ESR2* and *BNC2* that had unknown frequencies in the general population, together with a novel substitution in intron 2 of *NR5A1* (g.IVS2-5G>A) that was predicted to not affect splicing.

Copy-number analyses

One of the 62 patients (case 8) carried CNVs on the Y chromosome (Fig. 2A). These alterations consisted of copy-number gain of a ~23 Mb region from Ypter to Yq11.223 and copy-number loss of the remaining Y chromosomal region. The log2 signal ratios of most probes corresponding to the amplified and deleted regions were lower than +1.0 and higher than −2.0 respectively, indicating mosaicism of these CNVs. FISH analysis using a *SRY*-containing probe showed that case 8 had mosaic dicentric Y (Fig. 2B). CGH analysis for case 6 with an apparently homozygous *SRD5A2* mutation and case 7 with an apparently homozygous *HSD3B2* mutation excluded compound heterozygosity for a mutation and deletion (data not shown).

Clinical findings of patients with putative pathogenic defects

Putative pathogenic defects were associated with both anterior and posterior hypospadias (Table II). Endocrine evaluation of cases 1–8 remained fragmentary; blood hormone levels in cases 3 and 7 were within the normal range (Table II).

Discussion

Systematic mutation screening identified putative pathogenic mutations in 7 of 62 patients with non-syndromic hypospadias. These results, in conjunction with previous studies showing that ~30% of cases with severe hypospadias are ascribable to specific defects such as mutations in *AR* or *SRD5A2* (Albers *et al.*, 1997; Boehmer *et al.*, 2001), demonstrate the significant role of mutations in known causative genes in the etiology of non-syndromic hypospadias. Furthermore, our results support the previously proposed notion that genetic defects in *AR* account for a substantial percentage of cases with various types of 46,XY DSD (Albers *et al.*, 1997; Boehmer *et al.*, 2001; Audi *et al.*, 2010) and that mutations in *HSD3B2* can lead to non-syndromic hypospadias as a sole clinical manifestation, although *HSD3B2* plays an essential role in adrenal function (Boehmer *et al.*, 2001; Codner *et al.*, 2004; Audi *et al.*, 2010). Case 3 carried the p.R841S mutation in *AR*, which have been identified in patients with ambiguous genitalia (Melo *et al.*, 2003), suggesting the phenotypic diversity of missense mutations in *AR*. Notably, two of our patients had putative pathogenic mutations in multiple genes. Case 4 carried a hemizygous in-frame deletion/insertion in *AR* and a heterozygous missense substitution in *HOXB6*. Likewise, case 7 with a homozygous missense mutation in *HSD3B2* had an additional heterozygous missense mutation in *SRD5A2* that retains 3% of enzymatic activity (Makridakis *et al.*, 2000; Sasaki *et al.*, 2003). These data imply for the

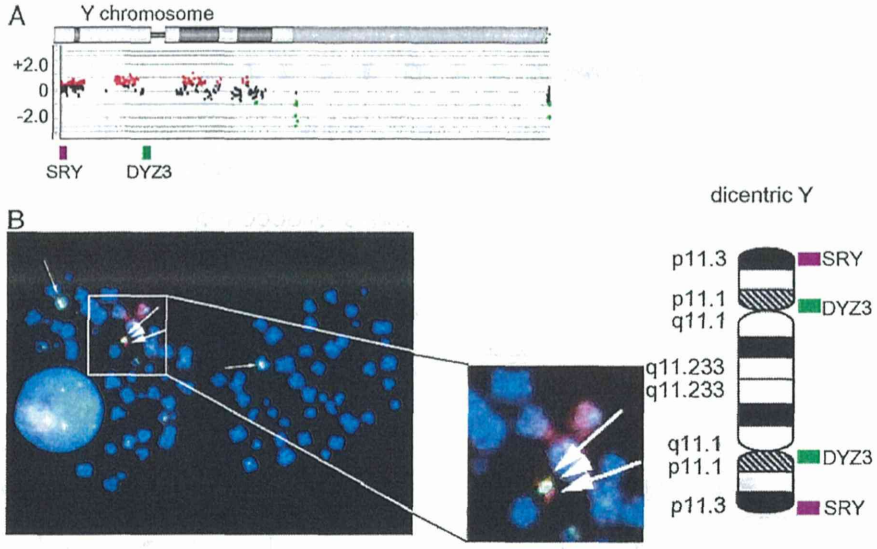

Figure 2 Copy-number alterations identified in case 8. Results of array-based comparative genomic hybridization (CGH) (**A**) and fluorescence *in situ* hybridization (FISH) analysis and schematic representation of the dicentric Y chromosome (**B**) are shown. The black, red and green dots in CGH denote signals indicative of the normal, increased (>+0.4) and decreased (<−0.8) copy-numbers, respectively. The arrowhead and thick arrows in FISH indicate a signal of DYZ3 (Y centromeric probe) and signals of *SRY*-containing probe (Yp11.3), respectively. The thin arrows in the left panel indicate signals of X centromeric probe.

Table II Molecular and clinical findings of patients with putative pathogenic abnormalities.

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8
Affected gene/region	*AR*	*AR*	*AR*	*AR/HOXB6*	*BNC2*	*SRD5A2*	*HSD3B2*	Y chromosome[a]
Ethnic origin	Japanese	Japanese	Japanese	Japanese	Japanese	Vietnamese	Vietnamese	Japanese
Family history of DSD	No	No data	No	No data	No	No	No	No
Clinical features								
Hypospadias[b]	Anterior	No data	Posterior	No data	Posterior	Posterior	Posterior	Posterior
Cryptorchidism	No	No data	No	No data	No	No	Yes (right)	No
Micropenis	No	No data	Yes	No data	No	Yes	Yes	No
Other features	No	No data	No	No data	No	No	No	Borderline MR
Endocrine findings								
Age at examination	No data	No data	15 months	No data	No data	No data	3.5 years	No data
LH (IU/l)[c]	No data	No data	<0.2 (<0.2–0.3)	No data	No data	No data	No data	No data
FSH (IU/l)[c]	No data	No data	<1.0 (<1.0–1.5)	No data	No data	No data	No data	No data
Testosterone (nmol/l)[c]	No data	No data	0.17 (0.10–0.45)	No data	No data	No data	0.16 (0.10–0.45)	No data

DSD, disorders of sex development; MR, mental retardation; LH, luteinizing hormone; FSH, follicle stimulating hormone.
[a]Copy-number alterations on Y chromosome.
[b]Position of urethral opening.
[c]Hormone values in parentheses indicate the reference ranges of age- and sex-matched control individuals.

first time that non-syndromic hypospadias results from digenic mutations. On the other hand, we did not observe the accumulation of rare SNPs in the patient group. Our data suggest that previously reported susceptibility SNPs play no or only minor roles in the development of non-syndromic hypospadias in the Japanese population. However, we cannot exclude the possibility that oligogenicity of these SNPs increases the risk of the disease, because a small number of our patients carried these SNPs as biallelic or digenic substitutions. Considering the small number of participants of this study, further investigations are necessary to clarify the possible association between rare SNPs and the disease phenotype.

Genome-wide copy-number analysis identified cryptic CNVs only in one patient. Case 8 carried a copy-number gain of a ~23 Mb region

on Yp and Yq and copy-number loss of the remaining Y chromosomal region. FISH analysis revealed that case 8 had mosaic dicentric Y, which has been described in multiple patients with hypospadias (Drummond-Borg *et al.*, 1988; Kojima *et al.*, 2001). It has been proposed that dicentric Y results in hypospadias by mosaic loss of the rearranged Y chromosome or by aberrant expression of Y chromosomal genes (Drummond-Borg *et al.*, 1988; Kojima *et al.*, 2001). The lack of pathogenic CNVs in the remaining 61 cases suggests the rarity of cryptic CNVs as genetic causes of non-syndromic hypospadias.

In this study, putative pathogenic defects were identified predominantly in patients with severe (posterior) hypospadias, while an *AR* mutation was detected in case 1, who manifested mild (anterior) hypospadias without micropenis or cryptorchidism. In this regard, previous studies have shown that syndromic hypospadias often arises from known gene mutations or chromosomal rearrangements (van der Zanden *et al.*, 2012). These data imply that monogenic mutations can underlie various types of hypospadias, although they are more strongly associated with severe or syndromic hypospadias than with mild non-syndromic hypospadias. Since identification of pathogenic defects can help to predict disease outcomes and improves the accuracy of genetic counseling, genetic analyses should be considered in patients with hypospadias of various clinical severities.

It should be pointed out that the present study has some limitations. First, the patient group consisted of only Japanese and Vietnamese individuals. Since the prevalence of hypospadias varies among countries (Nassar *et al.*, 2007; Serrano *et al.*, 2013), there may be ethnicity-specific causes of hypospadias. For example, mutations in *ATF3*, which account for ~10% of cases in the USA (Kalfa *et al.*, 2008), were absent from our cohort. In contrast, the p.A10T mutation in *HSD3B2* and the p.R227Q mutation in *SRD5A2* were detected exclusively in Vietnamese patients in homozygous state. Thus, our results are not simply applicable to other ethnic groups. Second, the frequency of monogenic defects may be underestimated in this study, because we focused on protein-altering mutations in 25 genes. Mutations/variations in regulatory regions, defects in unexamined genes and epigenetic abnormalities may be hidden in our mutation-negative patients. Lastly, clinical information of our patients remained fragmentary. Although previous studies have revealed that several factors such as low birthweight, placental insufficiency and maternal hypertension are associated with the risk of hypospadias (Stoll *et al.*, 1990; Weidner *et al.*, 1999; Fredell *et al.*, 2002; Brouwers *et al.*, 2010), the contributions of such factors to the disease phenotype of our patients are yet to be studied. Moreover, since endocrine data were unavailable for most of our mutation-positive cases, further studies are needed to elucidate the hormonal characteristics of each monogenic disorder.

Conclusion

The present study indicates that mutations in known causative genes and submicroscopic CNVs account for >10% of cases with non-syndromic hypospadias. Pathogenic defects appear to underlie both severe and mild hypospadias. On the other hand, previously reported risk SNPs are unlikely to play a major role in the development of the disease; further studies are required to validate this observation. Most importantly, this is the first report documenting the possible oligogenicity of non-syndromic hypospadias.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

Authors' roles

M.K., K.No., T.O., and M.F. designed the study. M.K., E.S., V.C.D., Y.H., T.M., K.Mu., K.U., N.I., K.Nag., Y.O., T.H., K.Y., M.I, Y.K.-F., K.Nak., K.Hay., K.Hat., Y.M., K.Mo., and T.O. contributed to the acquisition of data. M.K. and M.F. analyzed data and wrote the paper. All authors were involved in revising the paper and approved the final version of the manuscript for submission.

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Conflict of interest

The authors declare that there is no conflict of interest.

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The Journal of Immunology

Two Novel Gain-of-Function Mutations of *STAT1* Responsible for Chronic Mucocutaneous Candidiasis Disease: Impaired Production of IL-17A and IL-22, and the Presence of Anti–IL-17F Autoantibody

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Two Novel Gain-of-Function Mutations of *STAT1* Responsible for Chronic Mucocutaneous Candidiasis Disease: Impaired Production of IL-17A and IL-22, and the Presence of Anti–IL-17F Autoantibody

Yasuhiro Yamazaki,* Masafumi Yamada,* Toshinao Kawai,† Tomohiro Morio,‡ Masafumi Onodera,† Masahiro Ueki,* Nobuyuki Watanabe,† Hidetoshi Takada,§ Shunichiro Takezaki,* Natsuko Chida,*,¶ Ichiro Kobayashi,* and Tadashi Ariga*

Heterozygous gain-of-function (GOF) mutations of *STAT1* are responsible for chronic mucocutaneous candidiasis disease (CMCD), one of the primary immunodeficiency diseases characterized by susceptibility to mucocutaneous *Candida* infection. To date, 30 aa changes have been reported: 21 in the coiled-coil domain and 9 in the DNA-binding domain. In this study, we report two novel *STAT1* GOF mutations of p.K278E in coiled-coil domain and p.G384D in DNA-binding domain in Japanese CMCD patients. Ectopic expression of these STAT1 mutants in HeLa cells was associated with increased phosphorylation of the mutant and the endogenous wild-type STAT1 due to impaired dephosphorylation, indicating heterodimers of the wild-type and mutant STAT1 cause impaired dephosphorylation, as did homodimers of the mutants. Because IL-17A production was not significantly reduced at least in one of the patients following PMA plus ionomycin stimulation, we further studied Th17-associated cytokines IL-17A, IL-17F, and IL-22 in response to more physiologically relevant stimulations. IL-17A and IL-22 production from PBMCs and $CD4^+$ cells was significantly reduced in four patients with *STAT1* GOF mutations, including the previously reported R274Q in response to anti-CD3 plus anti-CD28 Abs or *Candida* stimulations. In contrast, IL-17F production was comparable to healthy controls in response to anti-CD3 plus anti-CD28 Abs stimulation. These results indicate impaired production of IL-17A and IL-22 rather than IL-17F was associated with the development of CMCD in these patients. Additionally, only the anti–IL-17F autoantibody was detected in sera from 11 of 17 patients with *STAT1* GOF mutations, which may be useful as a marker for this disease.
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Chronic mucocutaneous candidiasis (CMC) is characterized by susceptibility to *Candida* infection of the skin, nails, and mucosal membrane. CMC can occur as one of the various manifestations in patients with primary immunodeficiency diseases such as SCID, STAT3-deficient hyper-IgE syndrome (HIES), and autoimmune polyendocrine syndrome type 1 (APS1). Alternatively, CMC can be the only manifestation in patients with IL-17F deficiency, IL-17RA deficiency, or recently identified ACT1 deficiency (1, 2). This condition is described as isolated CMC or CMC disease (CMCD). Extensive analyses of CMCD patients resulting from impaired Th17 immunity have indicated the role of Th17 cells and Th17-associated cytokines in host defense against mucocutaneous *Candida* infection. In 2011, heterozygous *STAT1* mutations were reported as a cause of CMCD (3, 4), and since then, 30 aa changes have been reported: 21 in the coiled-coil domain (CCD) and 9 in the DNA-binding domain (DBD) (see Fig. 1A) (3–13). Accumulated data indicated these mutations account for around half of CMCD patients and were associated with gain of STAT1 function due to impaired dephosphorylation of STAT1 (4, 13). The development of CMCD in this disorder could be also attributable to impaired Th17 immunity (3, 4, 13, 14), although the precise mechanisms have not been elucidated.

In the present study, we describe two novel *STAT1* mutations of p.K278E and p.G384D in CCD and DBD, respectively, in three CMC patients. These mutations were associated with gain of STAT1 function due to impaired dephosphorylation of STAT1, but the proportion of $IL\text{-}17A^+$ cells was not significantly reduced in one of the patients after PMA plus ionomycin (IOM) stimulation. Thus, we further studied Th17-associated cytokines IL-17A, IL-17F, and IL-22 in response to more physiologically relevant stimulations (15) and found impaired production of IL-17A and IL-22 but not IL-17F. We also studied autoantibodies against Th17-associated cytokines in 17 patients with *STAT1* GOF mutations based on the facts that these patients manifest various autoimmune disorders (3, 4), and that patients with APS1 exhibiting various autoimmune disorders were shown to have neutral-

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Abbreviations used in this article: ANA, anti-nuclear Ab; APS1, autoimmune polyendocrine syndrome type 1; CCD, coiled-coil domain; CD3/28, anti-CD3 plus anti-CD28 Abs; CMC, chronic mucocutaneous candidiasis; CMCD, chronic mucocutaneous candidiasis disease; DBD, DNA-binding domain; EGFP, enhanced GFP; GOF, gain-of-function; HIES, hyper-IgE syndrome; ILC, innate lymphoid cell; IOM, ionomycin; IP10, IFN-γ–inducible protein 10; LCL, lymphoid cell line; rh, recombinant human; WT, wild-type.

izing autoantibodies against various Th17-associated cytokines that could be associated with the development of CMC (16, 17). Patients with *STAT1* GOF mutations were demonstrated to have anti–IL-17F autoantibody, although no neutralizing activity was observed.

Materials and Methods

Patients

Patient 1 is a 20-y-old woman born to nonconsanguineous healthy Japanese parents. Recurrent oral thrush developed since the age of 1 y. She also had recurrent herpes zoster more than five times since the age of 4 y, and recurrent stomatitis about once every 2 mo that persisted around a week every time. *Candida* esophagitis developed at the age of 18 y. Impetigo contagiosa on the right thigh developed at the age of 19 y.

Patient 2 is a 35-y-old woman who had suffered from repetitive oral thrush and stomatitis since infancy. She had atopic dermatitis treated by anti-allergy drugs and topical corticosteroid. Bronchiectasis developed at the age of 18 y. Esophageal stenosis possibly caused by *Candida* esophagitis developed at the age of 19 y, which required balloon dilatation for swallowing foods. Iron-deficiency anemia had developed since her late 20s. She suffered from herpes zoster infection three times at the age of 11, 30, and 33 y.

Patient 3 is a 5-y-old boy who is a son of patient 2. He had oral thrush and onychomycosis, and was diagnosed as having CMC at the age of 1 y. Herpes zoster infection developed at the age of 4 y.

Patients 1, 2, and 3 had normal proportion of lymphocytes and their subsets with normal levels of serum γ-globulins. These 3 patients had not presented with endocrine diseases or autoimmune diseases.

Patient 4 is a 14-y-old boy with a heterozygous GOF mutation of p.T385M affecting the DBD of STAT1 as described previously (13). We studied patient 4 as a control to study the phosphorylation state of STAT1 GOF.

Patient 5 is an 18-y-old woman with a heterozygous GOF mutation of p.R274Q affecting the CCD of STAT1 as described previously (13). We studied patient 5 as a control to analyze Th17-associated cytokine production.

Informed consent for genetic analysis was obtained from the patients as well as normal controls under a protocol approved by the Institutional Review Board of Hokkaido University Hospital.

DNA isolation, PCR, and sequence analysis of PCR products

These procedures were performed following the methods described elsewhere (18).

Generation of EBV-transformed cell lines

EBV-transformed cell lines (EBV-lymphoid cell lines [LCLs]) were generated by in vitro transformation of human B cells with EBV (strain B95-8) as described elsewhere (18). Based on the results of *STAT1* sequence analysis, EBV-LCLs from patients 1, 3, and 4 with the heterozygous mutations of p.K278E, p.G384D, and p.T385M were designated as K278E/wild-type (WT), G384D/WT, and T385M/WT, respectively. Two EBV-LCLs from healthy controls were designated as WT1 and WT2.

Measurement of IFN-γ–inducible protein 10 concentration in supernatant of EBV-LCLs using cytometric bead array

This procedure was performed following the methods described elsewhere (13).

Transient transfection of plasmids expressing STAT1–enhanced GFP WT or mutants into HeLa cells

To evaluate the phosphorylation state of the ectopically expressed STAT1 and the endogenous WT STAT1 separately, we prepared the constructs expressing STAT1–enhanced GFP (EGFP) fusion protein, which was made by inserting WT *STAT1* cDNA into the pEGFP-N1 vector (BD Biosciences, Franklin Lakes, NJ) multicloning site. We generated *STAT1* mutants with K278E, G384D, or T385M by mutagenesis (PrimeSTAR mutagenesis basal kit, TaKaRa Bio, Shiga, Japan) following the manufacturer's protocol. HeLa cells were stripped by trypsin treatment 7 h before transfection and replaced at a density of 2.5×10^5 cells/ml in six-well plates. Plasmid DNA (5 μg/plate) carrying the WT or the various mutants or without *STAT1* alleles was used for cell transfection with the TransIT-LT1 reagent (Mirus Bio, Madison, WI). HeLa cells stimulated with recombinant human (rh)IFN-γ or rhIFN-α 36 h after transfection were harvested by trypsin. Transfection efficiencies were evaluated by FACSCalibur on the basis of the $EGFP^+$ cells.

Studies of STAT1 phosphorylation state and staurosporine treatment of cells

A total of 1×10^6 cells/ml EBV-LCLs in RPMI 1640 with 10% FBS or transfected HeLa cells as described above were stimulated with 1:1000 diluted rhIFN-γ (Shionogi, Osaka, Japan) for 30 min, or 1500 U/ml rhIFN-α (BioSource International, Camarillo, CA) for 15 min in 5% CO_2 at 37°C. For staurosporine treatment, these cells were then incubated with 1 μM staurosporine (Alomone Labs, Jerusalem, Israel), the tyrosine kinase inhibitor, in 0.5% DMSO final concentration for 15 min successively. The cells were harvested, and nuclear extracts obtained as described previously (13) were subjected to immunoblot analysis.

Immunoblot analysis for the studies of STAT1 phosphorylation state

This procedure was performed basically following the methods described previously with minor modification (13). Briefly, the SNAP i.d. 2.0 protein detection system (EMD Millipore, Billerica, MA) was used for the detection of STAT1, p-STAT1, and lamin A based on the manufactures protocols. All of the primary Abs were used at 1:2500 dilution. HRP-conjugated anti-mouse IgG secondary Ab (GE Healthcare, Buckinghamshire, U.K.) was used at 1:2500 dilution. The blots were then visualized by Luminata Forte Western HRP substrate (EMD Millipore).

Analysis of the production of IL-17A, IL-17F, IL-22, IFN-γ, and IL-4 from PBMCs and $CD4^+$ cells

PBMCs were recovered by centrifuging blood samples on Ficoll gradients. $CD4^+$ cells were positively selected by magnet sorting using CD4 microbeads (human) (Miltenyi Biotec, Auburn, CA) from PBMCs following the manufacturer's instructions. They were then adjusted to 1×10^6 cells/ml in RPMI 1640 containing 10% FBS. For stimulation with anti-CD3 plus anti-CD28 Abs (CD3/28), 1×10^6 cells/ml PBMCs were stimulated with 25 μl Dynabeads Human T-Activator CD3/28 (Life Technologies, Oslo, Norway) for 72 h. For *Candida* stimulation, PBMCs were stimulated with 100 μg/ml *Candida* Ag for skin test (Torii Pharmaceutical, Tokyo, Japan) for 72 h. $CD4^+$ cells were stimulated with 25 μl Dynabeads Human T-Activator CD3/28 for 1×10^6 cells/ml for 72 h, and 400 ng/ml PMA and 10 μg/ml IOM for 6 h. The concentration of IL-17A, IL-17F, IFN-γ, and IL-4 in the supernatant was measured with cytometric bead array (BD Biosciences) following the manufacturer's instructions. IL-22 was measured with a human IL-22 Quantikine ELISA kit (R&D Systems, Minneapolis, MN). Data from triplicate independent experiments are reported as the means ± SD. The p values were calculated with a Mann–Whitney U test. The purity of $CD4^+$ cells was examined with FACSCalibur using anti-human PE-Cy5–conjugated anti-CD4 Ab (BioLegend, San Diego, CA).

Flow cytometric analysis of intracellular IL-17A expression in $CD4^+$cells

PBMCs at a density of 1×10^6 cells/ml were stimulated with 500 ng/ml PMA plus 5 μg/ml IOM for 6 h. We added of 10 μg/ml brefeldin A for the last 2 h. Harvested PBMCs were washed with TBST and stained with allophycocyanin-conjugated anti-human CD4 Ab (BioLegend) for 20 min. Cells were washed three times with PBS and fixed and permeabilized with a Foxp3 staining buffer set (eBioscience, San Diego, CA) for 20 min at 4°C. Cells were then washed three times and incubated for 30 min with PE-conjugated anti-human IL-17A (BioLegend). Cells were washed three times and analyzed with a BD LSRFortessa.

Detection of anti-cytokine autoantibodies in sera from patients by immunoblot analysis

Five hundred nanograms rhIL-17A, IL-17F, IL-22, IL-23, IL-1β, IL-6 (R&D Systems), IFN-α (BioSource International), and TGF-β1 (BioLegend) were separated by 15% polyacrylamide gels and transferred to Immobilon-P transfer membranes. The membranes were blocked by TBST with 5% non-fat skim milk for 1 h. They were then reacted with the sera at 1:1500 dilutions overnight at 4°C. The sera involved 17 STAT1 GOF CMC patients, 2 APS1 patients, 2 STAT3-deficient HIES patients, and 21 healthy controls. After washing four times with TBST, the membranes were incubated with HRP-conjugated goat anti-human IgG or IgA (Invitrogen, Frederick, MD) at 1:150,000 or 1:40,000 dilutions, respectively, in TBST with 5% non-fat skim milk for 1 h at 4°C. After washing four times, the blots were then visualized by ECL Select Western blotting detection reagent (GE Healthcare). To titrate anti–IL-17F autoantibody, we performed immunoblot analysis of sera from the patients serially diluted from 1:640 to 1:81,920.

Results

Two novel amino acid substitutions in STAT1

Direct sequence analysis demonstrated that patient 1 had a heterozygous base change of c.832A→G, p.K278E (K278E) in CCD, and the parents of patient 1 had WT sequence in *STAT1*. Both patients 2 and 3 had the heterozygous base change of c.1150G→A, p.G384D (G384D) in DBD of *STAT1* (Fig. 1A, 1B). These base changes have not been reported either as disease-causing mutations or as single nucleotide polymorphisms in the National Center for Biotechnology Information database, the Ensembl database, the Single Nucleotide Polymorphism Database, and the Human Genetic Variation Database, which have information on 1208 Japanese single nucleotide polymorphisms, or in our 100 controls without CMC (data not shown). Both K278E and G384D were evolutionarily conserved (Fig. 1C). K278E was predicted as tolerated by the sort intolerant from tolerant algorithm, and it was predicted as benign with a score of 0.011 (sensitivity, 0.96; specificity, 0.78) by the polymorphism phenotype-2 algorithm. G384D was predicted as tolerated by the sort intolerant from tolerant algorithm but was predicted as probably damaging by the polymorphism phenotype-2 algorithm with a score of 1.000 (sensitivity, 0.00; specificity, 1.00).

K278E and G384D were associated with STAT1 GOF and increased STAT1 phosphorylation after IFN-γ and IFN-α stimulation

Heterozygous *STAT1* GOF mutations were shown to be the genetic causes of autosomal dominant or sporadic CMC. The reported mutations were associated with increased STAT1 phosphorylation due to impaired dephosphorylation. First, to study whether the amino acid substitutions of K278E and G384D in STAT1 also lead to STAT1 GOF, the production of IFN-γ–inducible protein 10 (IP10), the downstream target of STAT1, was analyzed. IP10 production was significantly higher in K278E/WT and G384D/WT EBV-LCLs compared with healthy controls following IFN-γ stimulation (Fig. 2A). We then studied the STAT1 phosphorylation state in EBV-LCLs from the patients and controls. Expression of p-STAT1 following IFN-γ or IFN-α stimulation was higher in G384D/WT, K278E /WT, and T385M/WT than in WT1 EBV-LCLs (Fig. 2B, 2C). Ectopic expression of STAT1 mutants was associated with increased phosphorylation of the STAT1 mutants in HeLa cells (Fig. 2D). These results indicate that K278E and G384D are GOF mutations responsible for CMCD, and the mechanism of GOF is STAT1 hyperphosphorylation following the IFNs stimulations.

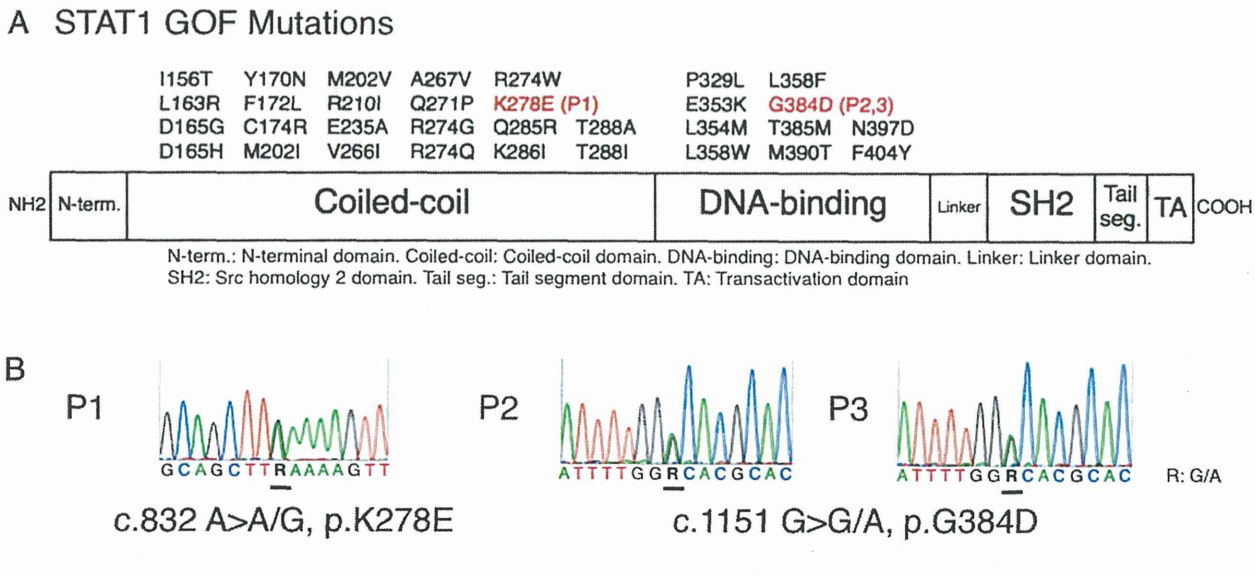

FIGURE 1. Two heterozygous base changes of *STAT1* leading to amino acid substitutions were identified in three CMCD patients. (**A**) The reported heterozygous *STAT1* mutations responsible for CMCD are shown. Mutations identified in this study are shown in red. (**B**) Direct sequence analysis of *STAT1* exon 10 in patient 1 (P1) and exon 14 in patients 2 (P2) and 3 (P3). Forward sequences are shown. (**C**) Comparison of the amino acid sequences of STAT1 in different species. The red rectangles indicate the amino acids corresponding to p.K278 or p.G384 in humans, respectively.

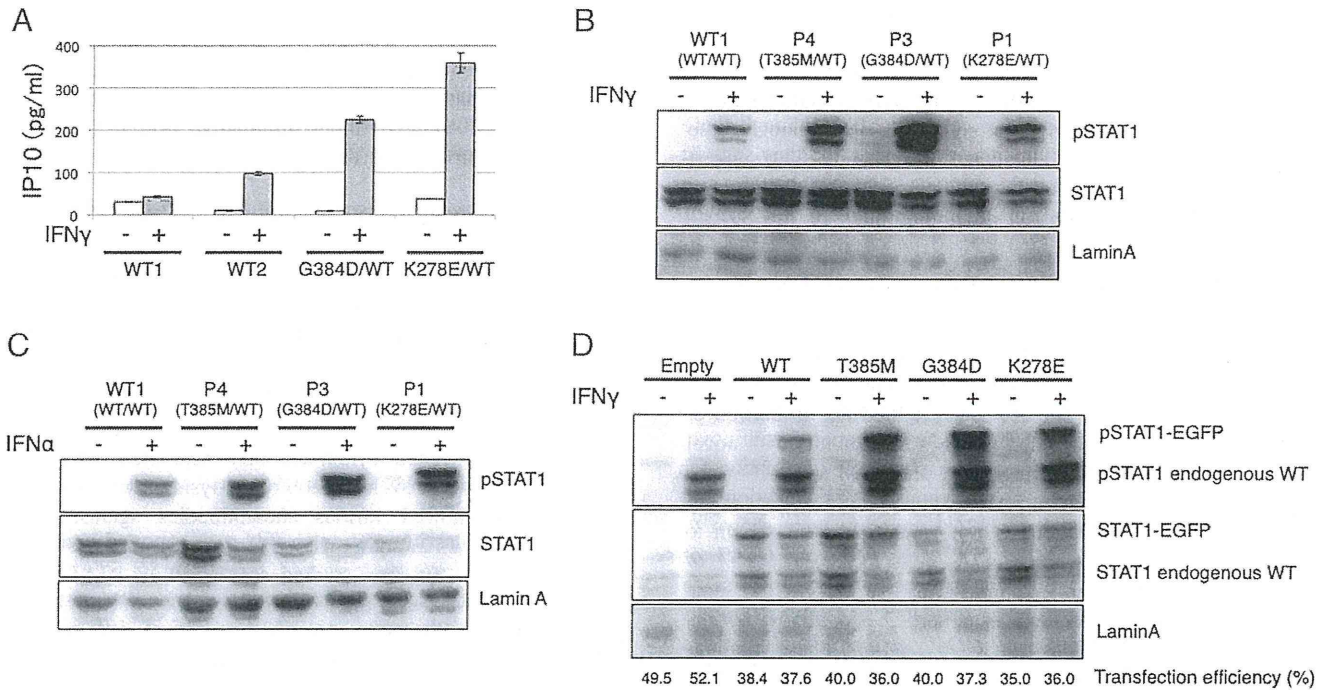

FIGURE 2. G384D and K278E were associated with GOF and increased phosphorylation of STAT1 in response to IFN-γ or IFN-α stimulation. (**A**) IP10 production from EBV-LCLs stimulated with or without IFN-γ for 6 h. (**B** and **C**) p-STAT1 and STAT1 expression in EBV-LCLs following IFN-γ stimulation for 30 min (B), and IFN-α stimulation for 15 min (C). (**D**) p-STAT1 and STAT1 expression in transfected HeLa cells following IFN-γ stimulation for 30 min. Transfection efficiencies were analyzed on the basis of GFP^+ cells and are described under each lane.

Increased phosphorylation of the STAT1 mutants was due to impaired dephosphorylation

The mechanisms underlying increased phosphorylation of the STAT1 mutants were further investigated with the tyrosine kinase inhibitor staurosporine. Dephosphorylation of the ectopically expressed STAT1-EGFP K278E or G384D mutants was impaired in HeLa cells following IFN-γ or IFN-α stimulation, compared with that of STAT1-EGFP WT (Fig. 3). Therefore, the mechanisms underlying increased STAT1 phosphorylation of K278E and G384D mutants involve impaired dephosphorylation of STAT1.

Increased phosphorylation and impaired dephosphorylation of the endogenous WT STAT1 in addition to the ectopically expressed mutants in HeLa cells

The endogenous WT STAT1 was also demonstrated to have increased STAT1 phosphorylation following IFN-γ stimulation when

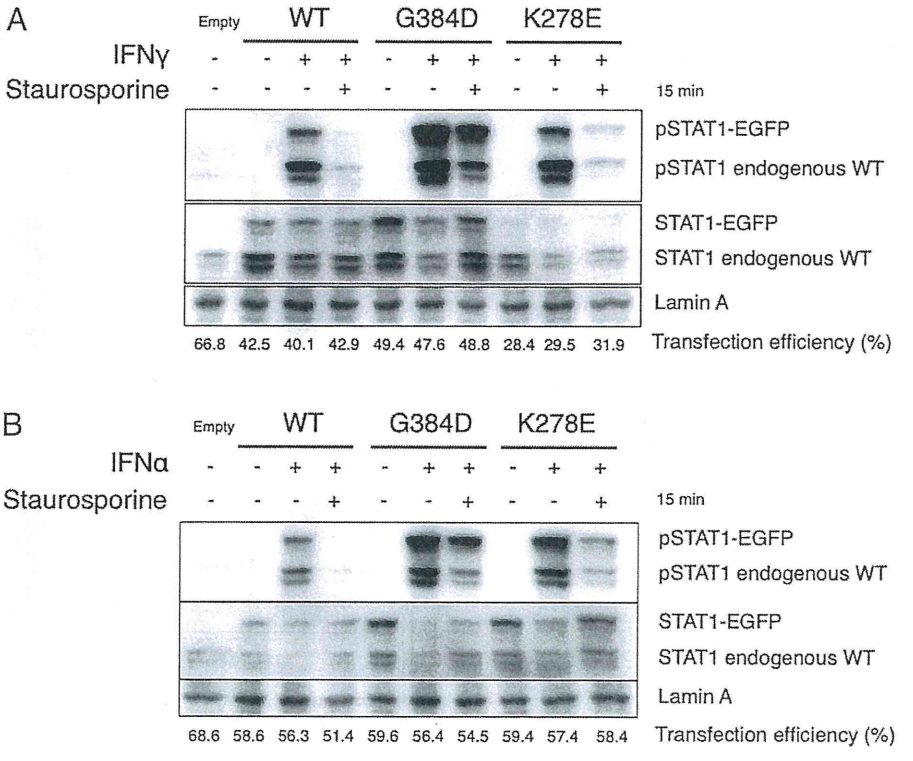

FIGURE 3. G384D and K278E were associated with increased phosphorylation of STAT1 due to impaired dephosphorylation. The p-STAT1 expression is shown in transfected HeLa cells first stimulated with IFN-γ for 30 min (**A**) or IFN-α for 15 min (**B**) followed by incubation with 1 μM staurosporine for 15 min. Transfection efficiencies are described under each lane.