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ORIGINAL ARTICLE

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Novel IRF6 mutations in Japanese patients with Van der Woude Syndrome: two missense mutations (R45Q and P396S) and a 17-kb deletion

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Abstract Three Japanese families with Van der Woude syndrome (VWS) were screened for mutations in the interferon regulatory factor 6 gene (*IRF6*) by sequencing its entire coding region. Two novel missense mutations, R45Q in exon 3 and P396S in exon 9, were identified in families 1 and 2, respectively. In family 3, no causative base change was found by the sequencing analysis, but a deletion involving exons 4–9 was suggested by multiplex PCR analysis. To confirm the deletion and to determine its 5′- and 3′-boundaries, we amplified a DNA fragment containing a heterozygous polymorphic site in exon 2 by using a 5′-upstream forward PCR primer and eight different reverse primers located 3′-downstream of exon 2. The amplified product was subjected to nested PCR to generate a DNA fragment containing the polymorphic site. When a reverse primer located within the deletion was used for the first PCR amplification, only the nondeletion allele was detected after the second PCR. Repeated analyses with eight different reverse primers allowed us to map the boundaries of the deletion, and subsequently a heterozygous 17,162-bp deletion involving exons 4–9 was identified. Since *IRF6* mutations in a significant portion of VWS patients remain undetected by conventional sequencing analysis, it may be important to search for a large deletion in those patients. Our simple methods to identify deletions and to determine the boundaries of a deletion would facilitate the identification of such patients.

Keywords Van der Woude syndrome (VWS) · Interferon regulatory factor 6 (IRF6) · Missense mutation · Large deletion · Multiplex PCR · Long distance PCR

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Introduction

Van der Woude syndrome (VWS, MIM no. 119300) is the most common syndromic clefting with an autosomal dominant trait characterized by congenital pits and sinuses in the lower lip, together with cleft lip or palate or both. Lip pits are depressions of the lower lip that contain the orifices of mucous glands or minor salivary glands. The incidence has been reported to be 1:75,000 live births (Cervenka et al. 1967), and it has been estimated to account for 2% of all cases of cleft lip and palate (Mutaf et al. 1993). The penetrance is 96.7% (Janku et al. 1980).

Bocian and Walker reported a large interstitial deletion at chromosome 1q32-q41 in a child with VWS. Chromosomal microdeletion has also been reported to cause VWS in a subset of patients (Bocian and Walker 1987; Houdayer et al. 2000; Sander et al. 1994; Schutte et al. 1999). An analysis of microsatellite markers has revealed a 350-kb critical region of VWS (Schutte et al. 2000). Recently, mutations in the interferon regulatory factor 6 gene (*IRF6*) were identified in patients with VWS, suggesting that haploinsufficiency of *IRF6* causes a disruption of orofacial development, which results in VWS (Kondo et al. 2002a; Shotelersuk et al. 2003). In contrast, dominant-negative mutations of *IRF6* disturb the development of the skin and genitalia as well as the face, and this is recognized as popliteal pterygium syndrome (PPS, MIM no. 119500). *IRF6* belongs to a family of nine

transcription factors that share a highly conserved helix-turn-helix DNA-binding domain and a less conserved protein-binding domain. Most IRFs regulate the expression of interferon alpha and interferon beta after viral infection, but the function of IRF6 remains unknown.

In this study, we performed a mutational analysis of three Japanese families with VWS by sequencing the entire PCR-amplified protein coding regions of *IRF6*. We found that two of three families had novel missense mutations. In the remaining family, a large deletion was suggested by multiplex PCR analysis. To confirm the deletion and to explore the 5′- and 3′-boundaries of the deletion, we devised a simple method using a nested PCR for efficient mapping of the deleted region.

Patients and methods

Families with VWS

Three Japanese families with VWS were recruited. This study was approved by the Ethics Committee of Tohoku University School of Medicine. Written and oral informed consent was obtained from all families. The phenotypic features of families 1 and 2 are shown in Fig. 1. The proband of family 1 had cleft lip and lip pits, while her father and her father's brother carried only lip pits (Fig. 1a). The phenotypic information of her grandparents was not available. Two affected sibs of family 2 had cleft lip, cleft palate, and lip pit (Fig. 1b). Their mother also had both cleft lip and palate. It was uncertain whether she had no lip pit at birth or whether the lip pit had been repaired. In family 3, the child presented with cleft lip, cleft palate, and lip pit. Both parents appeared asymptomatic at first glance. They had no cleft lip, cleft palate, or lip pit. However, thorough examination of the mother revealed that she had a submucous cleft palate without a bifid uvula.

Sequencing analysis of *IRF6* exons

DNA samples were prepared from whole blood using a GFX DNA purification kit (Amersham Pharmacia Biotech, Buckinghamshire, England). We amplified exons 1–9 using intronic PCR-amplification primers, as shown in Table 1. Each forward and reverse primer had additional M13 universal and reverse primer sequences at its 5′-end, respectively, which facilitated the direct sequencing analysis by the dye primer method as described (Kure et al. 1998).

Multiplex PCR analysis

We amplified multiple *IRF6* exons simultaneously by mixing PCR primer sets in combination with Multiplex PCR kit (Qiagen, GmbH, Germany). Each amplified PCR product was identified by 3% gel electrophoresis analysis. Multiplex PCR set A consisted of primer sets for amplifying exons 2, 3, 5 and 7; set B, exons 3, 4 and 6; set C, exons 2, 8, and 9; and set D, exons 1 and 2. The reaction mixtures (30 μl) contained 15 μl of 2× multiplex PCR buffer supplied by the manufacturer, 20 μM of each primer, and 100 ng of genomic DNA. The thermoprofile was 95°C for 15 min, 30 cycles of 95°C for 20 s, 57°C for 90 s, and 72° for 90 s, which was followed by 72°C for 10 min. A total of 10 μl of each PCR product was subjected to 3% agarose gel electrophoresis. The intensity of each band was recorded with a CCD camera and analyzed by Video In software (ATTO, Tokyo, Japan).

Determination of the boundaries of the deletion

We determined the 5′- and 3′-boundaries of the deletion by nested PCR amplification of a heterozygous polymorphic site followed by sequencing for allele discrimination. Long-distance PCR was performed as the first PCR using LA PCR Kit version 2.1 (Takara Biomedicals, Tokyo, Japan) according to the manufacturer's protocol. The thermocycle consisted of 94°C for 1 min, 30 cycles of 98°C for 20 s, and 68°C for 15 min, followed by 72°C for 10 min. The second PCR mixture with a total volume of 30 μl contained 0.05 μl of the first PCR product as a template and primers E2F and E2R (Table 1). The thermocycle conditions were 94°C for 1 min, 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, followed by 72°C for 10 min. The amplified products of the second PCR were directly sequenced as described above. A recombinant DNA fragment caused by the deletion was amplified using genomic DNA of the proband of family 3 and subjected to direct sequencing analysis.

Results

Sequencing analysis of each *IRF6* exon revealed two novel missense mutations in *IRF6* in families 1 and 2. In

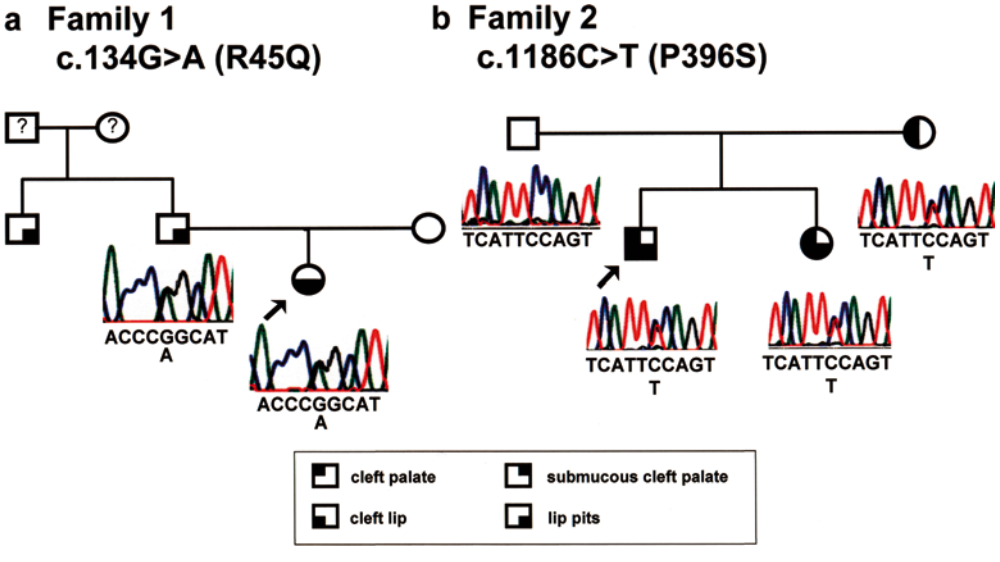

Fig. 1 The pedigrees of families 1 (**a**) and 2 (**b**) and identified *IRF6* mutations. Symbols representing specific phenotypes are shown in the *box*. The sequence chromatograms of the mutated sites are shown *underneath* each family member

Table 1 Primers for PCR

Primer name	Primer sequences [a]	Amplicons
Exon amplification and size of target		
E1F	5′-M13-AGAAGCGGAGGCGTAGGGTG-3′	573 bp (exon 1)
E1R	5′-R-ATCTGGAAAAGGGCGACAGG-3′	
E2F	5′-M13-TTATTCTAGGGCTTCTGAGC-3′	418 bp (exon 2)
E2R	5′-R-AAAGTTATGGAAACAGCAAC-3′	
E3F	5′-M13-GGCTAGAGCATGAAGTGTAA-3′	596 bp (exon 3)
E3R	5′-R-CATGCCCCCAAAAGAGGAAT-3′	
E4F	5′-M13-GCTCTGGGCCAATGATAGGAC-3′	409 bp (exon 4)
E4R	5′-R-AGGCTTTCTTGCTTTATCCA-3′	
E5F	5′-M13-CAGTGAATCTAGGGAGGTCC-3′	461 bp (exon 5)
E5R	5′-R-TGCTTTCAGGGCAGTGGTGG-3′	
E6F	5′-M13-CAGTGTTTGGTTCTTGTCTA-3′	468 bp (exon 6)
E6R	5′-R-TTTACTTCTTCCCTGGTGAC-3′	
E7F	5′-M13-AGTGGCCTTCCTGAATGCTG-3′	686 bp (exon 7)
E7R	5′-R-CTTGACCTCCTCCAGACTAA-3′	
E8R	5′-M13-AAAGATGGTATTTGTTGAGT-3′	472 bp (exon 8)
E8R	5′-R-GTTTCAGCAAGACTCTAAGG-3′	
E9F	5′-M13-ACATCAGAATGGGGTCTTCC-3′	588bp (exon 9)
E9R	5′-R-CATTAGGAGATTTGAAAAAG-3′	
Deletion mapping		
Forward primer	5′-CTCTTAGTACTTTAGTATTTGGTTATTCTAGGGC-3′	
r1	5′-CTCATCTCAGGAGAGGGAAGGAAAAAAGTTATGG-3′	
r2	5′-GAAGGTAATACCTCCCTTACAGGGTTGTCACG-3′	
r3	5′-R-TGCTTTCAGGGCAGTGGTGG-3′ [b]	
r4	5′-GACCGCTGACCTCCACCATCAGTCCTCTGTCCATG-3′	
r5	5′-GCATCATAGTGCACGACTGTTGTCCCAACTACTC-3′	
r6	5′-GCATTTGTGGCCAACTGATCTACAGCATTGTGCC-3′	
r7	5′-TGTGGAGTCCAAAGTCCTTCCCTAACGAAGTGGA-3′	
r8	5′-TCACTGTTTGAGCACCAACCATGTGCTGGGCAGTA-3′	
Detection of rearranged DNA		
4Fm13	5′-M13-GATGTTTCCCACCCAACTCAGT-3′	
215R	5′-R-AGTGCTGATGGTGGTGATTGCT-3′	

[a]M13 = TGTAAAACGACGGCCAGT, R = CAGGAAACAGCTATGACC
[b]Same as E5R in exon sequencing

family 1, a single nucleotide substitution from G to A at nucleotide position 134 of cDNA [c.134G > A, accession number (AC) NM_006147] was detected in exon 3 (Fig. 1a). This base change altered the second nucleotide of codon 45, resulting in an amino acid change from arginine to glutamine (R45Q). The same mutation was also detected in the proband's father, while the DNA sample of her mother was not available. In family 2, a single nucleotide substitution from C to T at cDNA position 1,118 (c.1186C > T) was detected in exon 9 of the proband (Fig. 1b), resulting in an amino acid substitution from proline to serine at position 396 (P396S). Family member analysis revealed that the mutation was found in both affected sibs and their mother, but not in their asymptomatic father. The R45Q and P396S substitutions were not observed in a total of 100 alleles from 50 control subjects (data not shown).

In family 3, sequencing analysis detected no causative base change. A single nucleotide polymorphism of C/T in exon 2 was observed in the proband, which has already been registered in NCBI SNP CLUSTER (ID rs861019). We carried out the multiplex PCR analysis to examine a possible deletion or duplication in *IRF6* (Fig. 2). Band intensities of exons 4–9 in the proband were lower than those in a control subject, while those of exon 1–3 were comparable, suggesting that one of two alleles of exons 4–9 was deleted in the proband.

We then studied further the deletion suggested in family 3. We devised a PCR-based method for determining the 5′- and 3′-boundaries of the deletion because the amount of the DNA sample was not sufficient for Southern blot analysis. The principle of the method is illustrated in Fig. 3. We took advantage of the C/T polymorphism in exon 2, which was located close to the deletion for allele discrimination. A long-distance amplification was used as the first PCR with forward (F1) and reverse primers (R1a, R1b or R1c) located upstream and down stream of the polymorphic site, respectively (Fig. 3a). The first PCR products were then subjected to a second PCR with nested primers (F2 and R2). When the reverse primer of the first PCR was located in the 5′-upstream region of the deletion (R1a), both A and B alleles were amplified in the second PCR and subsequently detected in the following sequencing analysis (Fig. 3b). When the reverse primer was located within the deleted region (R1b), only the nondeleted allele (B allele) was detected (Fig. 3c). If the reverse primer resides in the 3′-downstream region of the deletion (R1c), both A and B alleles were amplified in the first PCR or only A allele (deleted allele) was amplified when the distance between the two primers was too long to be amplified by PCR (Fig. 3d). Thus, the 5′-boundary could be mapped to the region between R1a and R1b, while the 3′-boundary was mapped to the region between R1b and R1c.

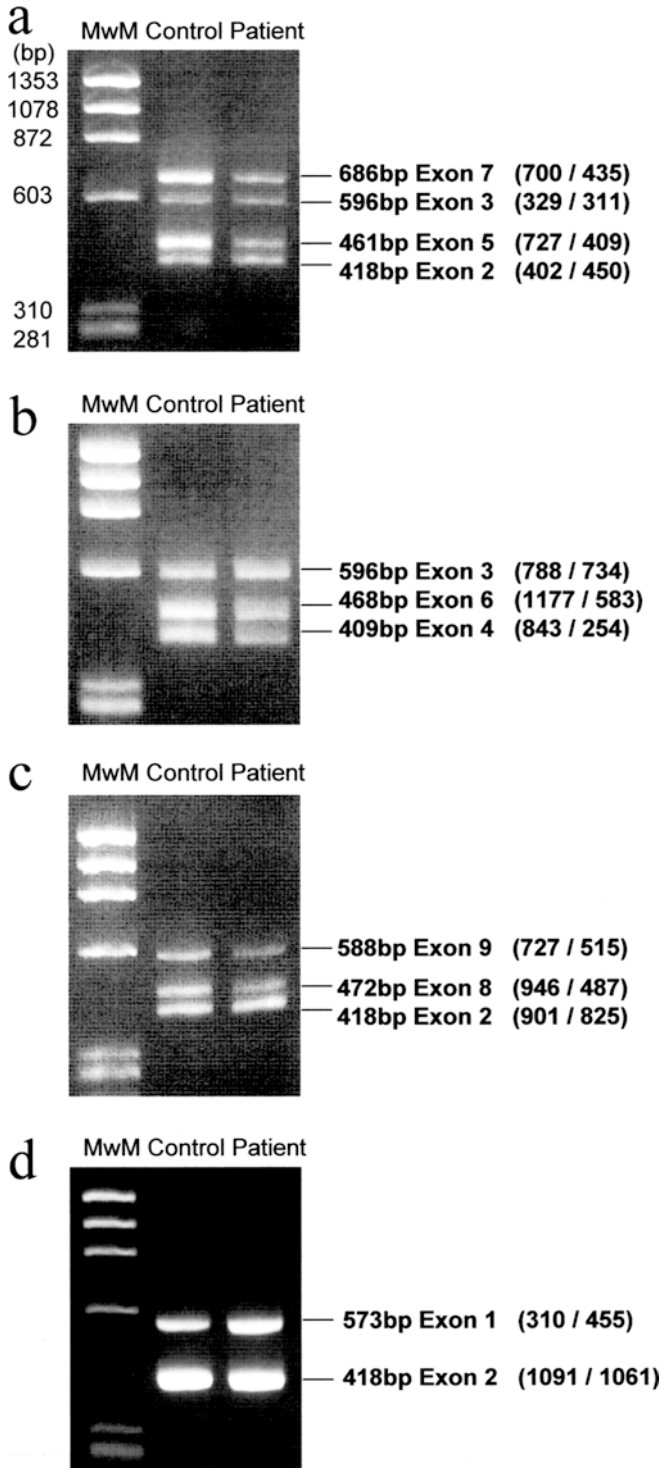

Fig. 2a–d Multiplex PCR analysis in family 3. The amplified DNA fragments and the band intensities of exons 2, 3, 5, and 7 are shown. **a** Exons 3, 4 and 6, **b** exons 2, 8, and 9, **c** exons 1 and 2, **d** relative band intensities expressed in arbitrary units are shown in *parentheses* (control/patient). *MwM* molecular weight marker

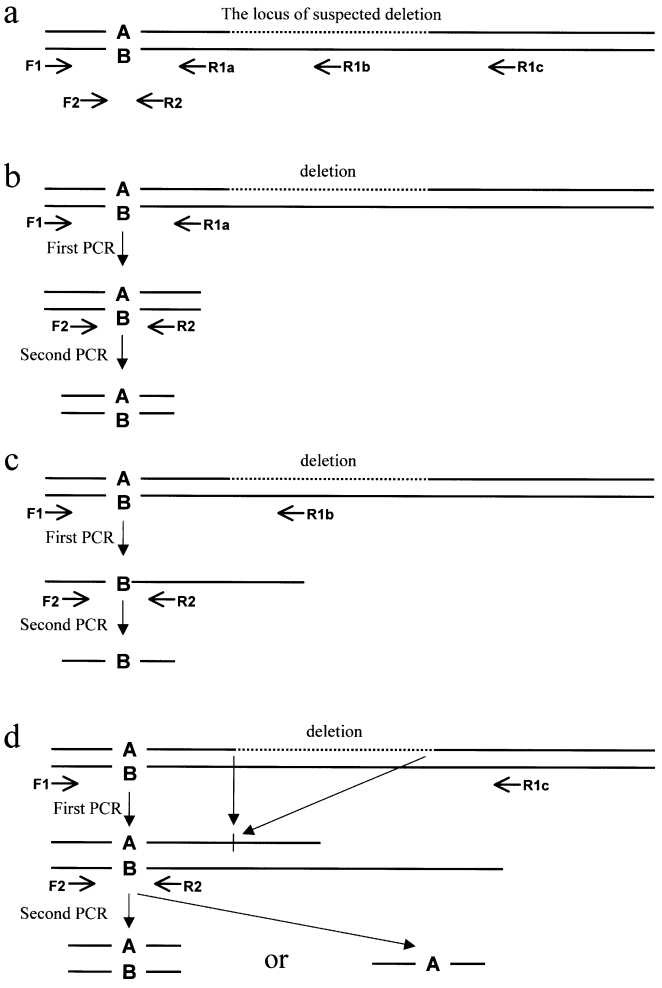

Fig. 3a–d Schematic drawing of a method for mapping deletion boundaries. The method takes advantages of a heterozygous polymorphic site for discriminating A and B alleles. A deleted region is shown as a broken line. **a** Positions of forward primers (F1 and F2) and reverse primers (R1a, R1b, R1c and R2) are shown. **b** When the reverse primer is located in the 5′-upstream region of the deletion, both alleles are amplified in the second PCR. **c** When the reverse primer is located within the deletion, only B allele, a nondeleted allele, is amplified. **d** When the reverse primer is positioned in the 3′-downstream region of the deletion, either both alleles are amplified or only A allele, a deleted allele, is amplified because the nondeleted allele may be too long to be amplified by PCR

One forward primer and eight reverse primers (r1–8) were designed for the first PCR (Table 1 and Fig. 4a). The r8 reverse primer was located in the 5′-upstream region of the polymorphic marker, *D1S3753*, which was previously used for the deletion analysis of a VWS family (Schutte et al. 1999). The *D1S3753* genotype of the proband was heterozygous, indicating that the deletion did not involve the *D1S3753* locus (data not shown). The primer set of the second PCR was the same as that for the sequencing analysis of exon 2. Both the C and T alleles were detected when primer r1 was used in the first PCR, whereas only the T allele was found when r2–6 primers were used. When the r7 and r8 primers were used, only the C allele was detected. These results suggested that the 5′-boundary of the deletion resided in the region between r1 and r2, while the region between r6 and r7 contained the 3′-boundary.

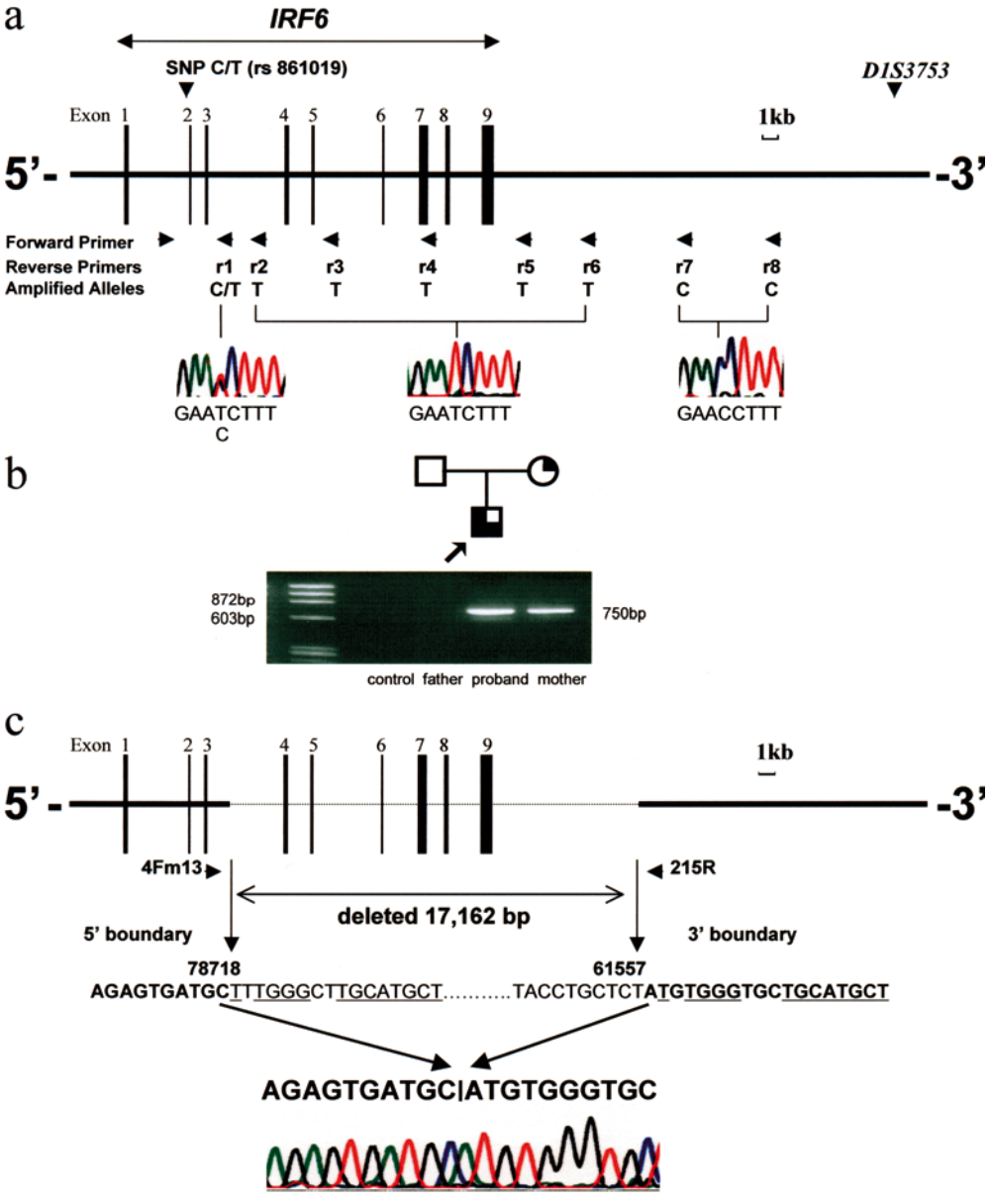

Fig. 4a–c Identification of the deletion boundaries in family 3. **a** Scanning of 5′- and 3′-boundaries of the deletion in the proband of family 3. Sequence chromatograms of second PCR products are shown. Both C allele and T allele were amplified when r1 primer was used in the first PCR. Only T allele was amplified when r2–r6 primers were used in the first PCR. Only C allele was amplified when r6 and r7 primers were used in the first amplification. Thus, we could predict that the 5′-boundary of the deletion was located between r1 and r2, while the 3′-boundary existed in the region between r7 and r8. Positions for two polymorphic markers, rs861019 and D1S3753, are indicated by *downward arrowheads*. **b** Detection of the rearranged fragment by PCR. Note that the rearranged DNA fragment of 750 bp was detected in the proband and his mother, but not in his father or in a control subject. Symbols representing specific phenotypes are the same as in Fig. 1. **c** Sequences of 5′- and 3′-boundaries of the deletion in the proband of family 3. Sequencing analysis of the rearranged DNA fragment shown in *panel b* revealed the 5′- and 3′-boundaries of the deletion. A 17,162-bp DNA fragment was deleted. The *underlined nucleotides* in the 5′-boundary were identical to that in the 3′-boundary

Based on these results, we amplified the deleted allele with the 4Fm13 primer and 215R primer using family 3 DNA as a template (Fig. 4c). The distance between the 4Fm13 and 215R primers was estimated to be 17,832 bp according to the sequence information of GenBank (AC AL022398.1). A 750-bp band was amplified in both the proband and his mother but not in his father or a control subject (Fig. 4b), indicating that recombination occurred within this fragment. The sequence analysis confirmed that a 17,162-bp DNA fragment from nucleotide position 78,718–61,557 was deleted (Fig. 4c).

Discussion

We screened three Japanese families with VWS for *IRF6* mutations and detected three novel mutations: a 17,162-bp deletion that included exons 4–9, R45Q in exon 3, and P396S in exon 9.

Kondo reported that sequencing analysis of each *IRF6* exon identified 55 mutations in 151 unrelated individuals with VWS (Kondo et al. 2002b). Mutations were not detected in a significant portion of the patients. This suggests a possible nonallelic heterogeneity in VWS or, alternatively, the presence of mutations undetectable by conventional sequencing analysis of exons and flanking introns. Such mutations may be nucleotide changes in promotor or intronic regions, deletion, duplication, or rearrangement. In fact, the mutation in family 3 was a large deletion involving exons 4–9. The deletion was not detected by our initial sequencing analysis. Chromosomal interstitial deletions (or microdeletions), which were detectable under a microscope after staining, have been reported in several VWS

families (Bocian and Walker 1987; Houdayer et al. 2000; Sander et al. 1994; Schutte et al. 1999). Small deletions within or around the *IRF6* gene, which were microscopically undetectable, also contributed to the disease, as demonstrated in this report.

The boundary sequences for the deletions reported in previous studies remain undetermined. We devised a novel PCR-based method for mapping boundaries of a deletion and successfully identified the boundary sequences using samples of less than 1 μg of genomic DNA. Deletions are often mediated by an aberrant recombination between multiple copies of similar or identical DNA sequences, for example, *Alu* repeats or L1 repeats (Suminaga et al. 2000).

A mechanism of unequal crossing over is believed to be responsible for the deletion of one of the alpha-globin genes in alpha-thalassemia deletion. In our study, no *Alu* repeats or L1 repeats were found in the vicinity of the boundaries, but a 16-bp sequence at the 5′-boundary of the deletion resembled a 17-bp sequence at the 3′-boundaries (Fig. 4c): '*TTTGGG*CT*TGCATGCT*' at the 5′-end of the deletion and '*TGTGGG*TGC*TGCATGCT*' at the 3′-end of the deletion. These similar sequences may have caused recombination and mediated the 17-kb deletion.

Neither a R45Q nor P396S missense mutation was detected in 100 control alleles, and they are not registered in the database of Japanese Single Nucleotide Polymorphisms (JSNP, http://snp.ims.u-tokyo.ac.jp/index_ja.html). Therefore, these nucleotide changes are unlikely to be polymorphisms. IRF6 has two conserved domains, a helix-turn-helix DNA-binding domain (amino acids 13–113) and a protein-binding domain (amino acids 226–394) termed SMIR (Smad-interferon regulatory factor-binding domain). Most of the previously identified missense mutations in *IRF6* (35 out of 37) are localized to regions encoding these two domains (Kondo et al. 2002a). The R45Q mutation is localized in the DNA-binding domain, whereas the P396S mutation is not localized in either of the two domains.

Family analysis of an *IRF6* mutation facilitates the calculation of the correct recurrence risk for genetic counseling of families with VWS. Minor manifestations of VWS include submucous cleft palate, missing teeth, and single lip pit. A parent with one of these phenotypes may be misdiagnosed as "unaffected," and the recurrence risk in the family may be underestimated. Phenotypic variations of patients even with the identical mutation also increase difficulties in estimating the recurrence risk. (Janku et al. 1980; Kondo et al. 2002a). It is of note that risks to offspring of affected parents are 42.7% for lip pits without clefts and 11% for clefts with or without lip pits (Janku et al. 1980). In family 3, the mother had only a submucous cleft palate. She did not have bifid uvula, which is often associated with VWS and provides a hint for the correct diagnosis for VWS (Abyholm, 1976). While careful physical examination to find any faint manifestation of VWS is important in the family analysis, the genetic testing will be a powerful tool to confirm the disease status in family members.

In conclusion, we identified two novel missense mutations, R45Q and P396S, and a 17,162-bp deletion in *IRF6* in patients with VWS. This observation indicates that it would be indispensable to look for a large gene deletion in the DNA diagnosis of VWS when no nucleotide substitution is found by conventional sequencing analysis. Our methods to identify deletions and to determine the boundaries of a deletion would facilitate the identification of such patients.

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