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Mutations of CLCN5 in Japanese children with idiopathic low molecular weight proteinuria, hypercalciuria and nephrocalcinosis

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Mutations of CLCN5 in Japanese children with idiopathic low molecular weight proteinuria, hypercalciuria and nephrocalcinosis. The annual urinary screening of Japanese children above three years of age has identified a progressive renal tubular disorder characterized by low molecular weight proteinuria, hypercalciuria and nephrocalcinosis. The disorder has been observed in over 60 patients and has a familial predisposition. Mutations of a renal chloride channel gene, CLCN5, have been reported in four such families, and we have undertaken studies in additional patients from 10 unrelated, non-consanguineous Japanese families to further characterize such CLCN5 mutations and to ascertain their prevalence. CLCN5 abnormalities were identified in 7 of the 10 unrelated patients and consisted of 5 mutations (2 nonsense, 1 frameshift and 2 missense), 1 deletion and 1 silent polymorphism. A clustering of these mutations in CLCN5 exons 8 and 10 was observed. Over 80% of the CLCN5 mutations could be readily detected by single stranded conformational polymorphism (SSCP) analysis, thereby providing a useful mutation screening method. Our results, which indicate that over 70% of Japanese patients with this renal tubulopathy have CLCN5 mutations, will help in the genetic and clinical evaluation of children at risk from this disorder.

The annual urinary screening program of Japanese children above three years of age has defined a progressive proximal renal tubular disorder characterized by low molecular weight proteinuria (such as β_2 microglobulinuria, normal child < 320 μg/liter, normal adult < 250 μg/liter), hypercalciuria and nephrocalcinosis [1]. Hematuria, glycosuria, aminoaciduria, an impaired urinary concentrating ability and mild renal impairment are also associated features that may occur in some children [1–4]. The disorder, which occurs predominantly in males, has been reported to have a familial predisposition and recent studies of four such Japanese kindreds have demonstrated an association with mutations of the X-linked renal chloride channel gene, CLCN5 [5]. The human CLCN5 gene, which is located on chromosome Xp11.22, consists of at least 12 exons that span 25 to 35 Kb of genomic DNA and encodes a 746 amino acid protein [6, 7]. CLCN5 belongs to a family of voltage-gated chloride channel genes (CLCN0, CLCN1 to CLCN7, and CLCNKa and CLCNKb) that encode proteins (CLC-0, CLC-1 to CLC-7, and CLC-Ka and CLC-Kb, respectively) that have about 12 transmembrane domains (Fig. 1) [6, 8, 9]. These chloride channels have an important role in the control of membrane excitability, transepithelial transport and possibly cell volume [8]. Heterologous expression of wild-type CLCN5 in *Xenopus* oocytes has revealed that the channel, CLC-5, conducts chloride currents that are outwardly rectifying and time-independent [5, 10, 11], and similar expression of CLC-5 mutants that caused this renal tubulopathy were associated with either markedly reduced or absent currents [5, 10]. The CLCN5 mutations observed in the Japanese patients were similar to those observed in the renal tubular disorders of Dent's disease, X-linked recessive nephrolithiasis (XRN) and X-linked recessive hypophosphatemic rickets (XLRH) [5, 10]. Dent's disease, XRN and XLRH, which have been described in British, North American and Italian families, respectively, are X-linked renal proximal tubulopathies that are characterized by low molecular weight proteinuria, hypercalciuria, nephrocalcinosis, nephrolithiasis and renal failure [12–16]. Thus, these disorders have marked phenotypic and genotypic similarities and we propose to refer to these variants collectively as Dent's disease. The occurrence of the Japanese variant, referred to as Dent's$_{JAPAN}$, has been reported in more than 60 patients in Japan, and to date CLCN5 mutations have been reported in four kindreds only [5]. Thus, in order to ascertain the prevalence of CLCN5 mutations in Dent's$_{JAPAN}$, we have undertaken investigations in 10 other Japanese kindreds, using the combined methods of single stranded conformational polymorphism (SSCP) analysis and direct DNA sequence analysis [5, 10, 17].

Key words: chloride channel gene, low molecular weight proteinuria, hypercalciuria, nephrocalcinosis.

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METHODS

Patients

Fourteen children from ten unrelated, non-consanguineous Japanese families who were identified from the annual urinary screening program to have β_2 microglobulinuria (normal child $<$ 320 μg/liter) were clinically and biochemically assessed for idiopathic low molecular weight proteinuria, hypercalciuria and nephrocalcinosis, as previously described [1]. The details of the 14

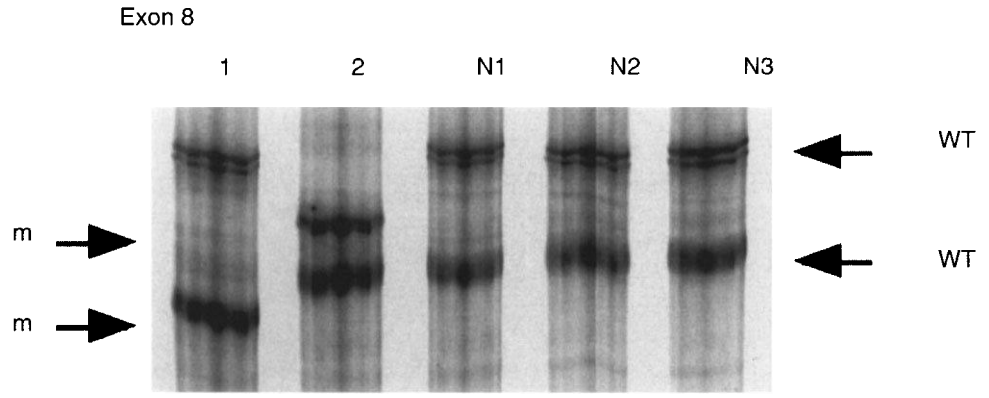

Fig. 1. Detection of two CLCN5 mutations in exon 8 by SSCP analysis. The results of SSCP analysis of the R347X (Lane 1) and W279X (Lane 2) mutations (Table 2 and Fig. 2) detected in the Dent's$_{JAPAN}$ families 10/95 and 20.1/95, respectively, together with three unrelated normals (N1 to N3) are shown. The mutant bands (m), Lanes 1 and 2, differed from the wild-type (WT) bands. These SSCP results were obtained by use of CLCN5 gene specific primers for exon 8, and 12.5% gels run at 15°C and at 350 volt-hours, as previously described [5].

Table 1. Clinical and biochemical findings in 14 Dent's$_{JAPAN}$ patients from 10 families

Family	13.1/95	19/95	15/95	9.3/95		10/95	9.2/95	21/95		6/95		20.1/95	20.2/95		Normal
Patient	1	2	3	4	5	6	7	8	9	10	11	12	13	14	child (adult)
Age *years*	14	13	10	11	8	7	12	17	13	20	13	17	7	5	
Sex	F	M	M	M	M	M	M	M	M	M	M	M	M	F	
Urine															
Protein	1+	1+	3+	2+	1+	2+	±	1+	2+	1+	1+	2+	1+	±	—
β2-microglobulin *μg/liter*	22400	28000	99000	76483	28681	130000	10000	65300	129000	65000	45000	97000	20000	16000	<320(<250)
Occult blood	—	—	1+	1+	1+	±	±	±	—	±	±	1+	—	—	—
Ca/Cr %	39	31	NT	37	30	NT	8	27	28	19	41	27	4	11	<25
Serum															
Creatinine *mg/100 ml*	0.5	0.5	1.0	0.6	0.6	0.3	0.6	1.1	0.5	0.7	0.5	0.7	0.3	0.7	0.1–0.8(0.6–1.5)
Cl *mEq/liter*	102	108	107	103	107	109	102	103	109	102	104	101	108	107	100–108
Ca *mg/100 ml*	10.2	9.0	9.2	9.4	10.1	9.8	9.6	9.7	9.7	9.2	9.8	9.5	10.3	9.4	9.0–10.6
P *mg/100 ml*	5.1	3.9	3.8	4.9	4.9	5.8	5.6	4.2	5.0	5.0	6.0	4.3	5.0	4.6	3.8–5.6
Nephrocalcinosis															
Ultrasonography	+	+	−	+	+	NT	−	−	−	NT	−	−	−	−	—
CT	+	+	NT	+	+	−	+	−	−	NT	+	−	−	−	—

Abbreviations are: Ca, calcium; Cr, creatinine; Cl, chloride; P, phosphate; CT, computerized tomography; +, present; −, absent; NT, not tested. All values refer to those obtained on early morning samples after an overnight fast.

Glycosuria was absent, and serum concentrations of sodium, potassium, urate and magnesium, and blood urea nitrogen were normal in all individuals. β_2 microglobulinuria (> 250 μg/liter) was present in the 4 mothers of the affected children from families 19/95, 9.3/95, 10/95 and 20.1/95.

affected children from these 10 families designated 6/95, 9.2/95, 9.3/95, 10/95, 13.1/95, 15/95, 19/95, 20.1/95, 20.2/95, 21/95, are summarized in Table 1. The clinical and biochemical details obtained five years earlier from patient 2 of family 19/95 have been previously reported as patient 3 [1]. Urinary samples for assessment of β_2 microglobulin were available from the mothers of the affected children in 7 of the 10 families–13.1/95, 19/95, 9.3/95, 10/95, 9.2/95, 20.1/95 and 20.2/95 (Table 1)–and the concentration of urinary β_2 microglobulin (normal adult < 250 μg/liter) in these mothers was 180, 2,400, 2,000, 980, 220, 1,400 and 210, respectively. Venous blood samples were obtained from 15 (14 patients in Table 1, and one mother from family 9.3/95) of the 18 affected members (14 patients in Table 1, and the 4 mothers with β_2 microglobulinuria from families 19/95, 9.3/95, 10/95 and 20.1/95) and used for mutational analysis of the CLCN5 gene [10].

DNA sequence analysis of the CLCN5 gene

DNA sequence abnormalities were initially sought for using single stranded conformational polymorphism (SSCP) analysis. Leukocyte DNA was extracted from the affected family members and used with CLCN5 specific primers for PCR amplification utilizing conditions previously described [5]. The PCR products were analyzed for SSCPs using the Phast electrophoresis system (Pharmacia, LKB, Uppsala, Sweden) as reported previously using the method of silver staining with 0.025 M aqueous silver nitrate for 10 minutes to reveal the SSCPs [5, 17]. Genomic DNA samples from 10 unrelated normal individuals were also used as controls in the SSCP analysis. The DNA sequence of abnormal SSCPs was determined by the use of *Taq* polymerase cycle sequencing and a semi-automated detection system (ABI 373A sequencer; Applied Biosystems, Foster City, CA, USA) [5, 17]. In addition, these DNA sequence abnormalities were confirmed either by restriction endonuclease analysis of genomic PCR products obtained by use of the appropriate primers or by sequence specific oligonucleotide (SSO) hybridization analysis [10, 17, 18]. These DNA sequence abnormalities were also demonstrated to be absent as common polymorphisms in the DNA obtained from 56 unrelated normal Japanese individuals (54 females, 2 males) and 75 unrelated normal European individuals (40 males and 35 females). Southern

Table 2. CLCN5 mutations in Dent's$_{JAPAN}$ families

Family	Codon	Base change	Amino acid ($\alpha\alpha$) change	Restriction enzyme change/SSO	Detected by SSCP[a]	Predicted effect
Nonsense mutations						
20.1/95	279	TGG→TGA	Trp→Stop	MaeIII	Y	Loss of 469 amino acids from D6 to C terminus
10/95	347	CGA→TGA	Arg→Stop	SSO	Y	Loss of 399 amino from D8 to C terminus
Missense mutations						
19/95	513	GGG→GAG	Gly→Glu	MnlI	Y	Disruption of charge distribution within D11
6/95	516	CGG→TGG	Arg→Trp	HinfI	N	Disruption of charge distribution within D11
Frame shift mutation						
15/95	229	del C, ins TA	7 missense $\alpha\alpha$ and stop	SSO	Y	Missense peptide from amino acids 229-235 and loss of 510 amino acids from D5 to C terminus
Deletion						
9.3/95	>180 kb deletion					Absence of protein
Polymorphism						
20.2/95	484	ACC→ACT	No change	AvaII	Y	No change

[a] Y, yes; N, no

blot hybridization analysis was used to assess genomic deletions of CLCN5 as described previously [10, 12].

RESULTS

SSCP analysis of the entire 2238 bp coding region of the CLCN5 gene from one affected member of each of the 10 unrelated, non-consanguineous Dent's$_{JAPAN}$ families (Table 1) revealed the presence of abnormal bands (Fig. 1) in the five families, designated 20.1/95, 10/95, 19/95, 15/95 and 20.2/95 (Table 2). In addition, PCR products could not be obtained, using any of the CLCN5 specific primers, from the DNA of the patient from family 9.3/95, indicating the presence of a genomic deletion of CLCN5 in this patient. Thus, CLCN5 abnormalities were detected by SSCP analysis in 60% of the Dent's$_{JAPAN}$ families. The nature of these SSCP abnormalities was further determined by DNA sequence analysis, which revealed the presence of four mutations that consisted of two nonsense (W279X, R347X), 1 missense (G513E) and 1 insertional-deletional mutation resulting in a frameshift (229fs) mutation, and one silent polymorphism (T484T) (Table 2 and Fig. 2). In addition, DNA sequence analysis of the entire 2238 bp coding region of the CLCN5 gene was performed in the patients from the four families (6/95, 9.2/95, 13.1/95 and 21/95) in whom SSCP abnormalities were not detected. This revealed the presence of one additional missense mutation (R516W) (Fig. 3) that had not been detected by SSCP analysis (Table 2). Thus, CLCN5 abnormalities consisting of 5 mutations, 1 deletion and 1 polymorphism (Table 2 and Fig. 2) were detected in 70% of the 10 Dent's$_{JAPAN}$ families. Two of these mutations (W279X and R347X) from families 20.1/95 and 10/95, respectively, occurred in exon 8; another two mutations (G513E and R516W) occurred in exon 10, which encodes the putative transmembrane domain 11 (D11) (Fig. 2). One mutation (229fs) occurred in exon 6 and the polymorphism (T484T) occurred in exon 9. The W279X mutation resulted from a G to A transition (TGG→TGA), the R347X resulted from a C to T transition (CGA→TGA), the G513E mutation resulted from a G to A transition (CGG→GAG), the R516W mutation resulted from a C to T transition (CGG→TGG), the 229fs mutation resulted from a deletion of one base pair (C) and an insertion of two base pairs (TA), and the polymorphism T484T resulted from a C to T transition (ACC→ACT). The W279X, G513E and R516X mutations, and the T484T polymorphism resulted in an alteration of a restriction enzyme site (Table 2) that facilitated its detection and confirmation (Fig. 3). The R347X and 229fs mutations were not associated with an altered restriction enzyme site and the method of *s*equence *s*pecific *o*ligonucleotide (SSO) hybridization was used to facilitate their detection and confirmation (Table 2). The extent of the CLCN5 deletion in the affected members of family 9.3/95 was assessed by Southern blot hybridization analysis and PCR using the genetic markers L(F1001), DXS255 (M27β), DXS146 and DXS988 [6, 12]. The loci DXS255 (M27β) and L(F1001), which are within a 180Kb region [6], were deleted whereas the loci DXS146 and DXS988, which are more than 2 Mbp in the telomeric and centromeric directions, were present (data not shown). Thus, the genomic deletion in family 9.3/95 encompassed CLCN5, DXS255 (M27β) and L(F1001) and indicated that this deletion was greater than 180 Kb in size. The absence of each of these 7 DNA sequence abnormalities (5 mutations, 1 deletion and 1 polymorphism) in 220 alleles from 130 unrelated normal individuals (56 Japanese and 75 Northern Europeans) established that these abnormalities were not common sequence polymorphisms that would be expected to occur in >1% of the population.

DISCUSSION

Our results, which have identified 5 mutations, 1 deletion, and 1 silent polymorphism of the CLCN5 gene in Japanese families with low molecular weight proteinuria, hypercalciuria and nephrocalcinosis (Dent's$_{JAPAN}$), expand the spectrum of such mutations associated with this proximal renal tubulopathy. Each of the five mutations (229 fs, W279X, R347X, G513E and R516W) and the CLCN5 deletion predicts a structurally significant alteration to CLC-5 (Table 2 and Fig. 2) and is thus likely to be of importance in the etiology of the disease. The two nonsense mutations (W279X and R347X), and the frameshift mutation that results in a termination at codon 235, predict truncated CLC-5 channels that lack respectively the 469 amino acids from D6 to the C-terminus, the 399 amino acids from D8 to the C-terminus, and the 510 amino acids from D5 to the C-terminus. The effects upon CLC-5 function of such nonsense mutations, which have been previously assessed [10], are likely to be a loss of function. The two

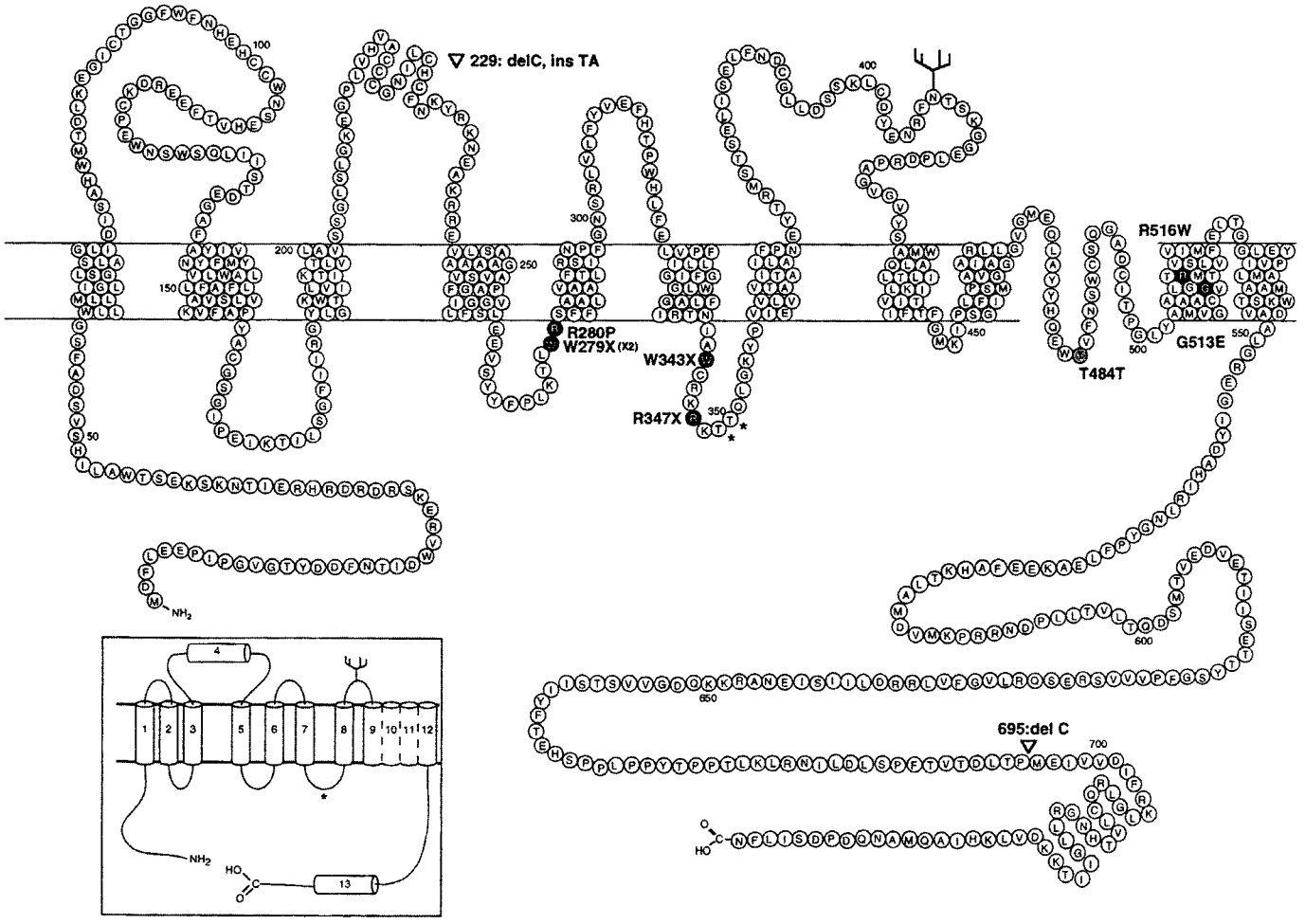

Fig. 2. Schematic representation of the Dent's$_{JAPAN}$ mutations within the framework of the predicted topology of CLC-5, which consists of 746 amino acids [6, 10]. The correct topology of the CLC-5 putative transmembrane domains (D1 to D13) is based upon a model [8, 10, 19] (*inset*) that places D4 extracellularly, and in which the hydrophobic core of the D9 - D12 crosses the membrane 3 or 5 times and contains a hydrophilic region whose precise location remains unkown. The consensus phosphorylation and glycosylation sites are indicated by asterisks and branch signs, respectively. The four previously reported CLCN5 mutations (W279X, R280P, W343X and ▽695:Cdel) [5] in Dent's$_{JAPAN}$ patients and the 5 additional CLCN5 mutations (R347X, W279X, G513E, R516W and the deletional—insertional frameshift mutation at codon 229 (del C, ins TA) detected by the present study are shown. The location of a silent polymorphism at T484T, which is unlikely to alter CLC-5 function as it is not associated with an altered amino acid in the channel, is also illustrated. In addition to these 9 mutations and one polymorphism, a microdeletion (Table 2) that was > 180 Kb in size and which encompassed the entire CLCN5 gene was found in one Dent's$_{JAPAN}$ family (9.3/95, Table 1). Thus, of the 14 Dent's$_{JAPAN}$ families studied to date, functionally significant CLCN5 abnormalities have been identified in 10, representing > 70%, of the kindreds.

missense mutations (G513E and R516W) also predict significant changes to CLC-5. Thus, the mutation of codon 513 results in the replacement of an uncharged amino acid (glycine, G) for a negatively charged amino acid (glutamate, E), thereby leading to a disruption of charge distribution within the putative transmembrane domain D11 (Fig. 2). Similarly, the mutation of codon 516 results in the replacement of a charged amino acid (arginine, R) for an uncharged, non-polar, hydrophobic amino acid (tryptophan, W), and this again is likely to lead to a disruption of charge distribution within D11 (Fig. 2). The effects of such missense mutations involving the highly conserved D11 [6], for example G506E, G512R and S520P, upon CLC-5 electrophysiological function, using the *Xenopus* oocyte expression system, have been previously demonstrated to result in either a complete loss or a marked reduction in the biological activity of CLC-5 [10, 19, 20]. It is thus highly probable that the *in vitro* electrophysiological effects of the disease-causing (that is, *in vivo* effects) mutations G513E and R516W would be similar, and such *in vitro* studies of the mutant CLC-5 genes were therefore not undertaken. A correlation between the different mutations (Table 2 and Fig. 2) and the severity of the phenotype (Table 1) could not be established, and the patients with the nonsense mutations, the missense mutations, and those in whom CLCN5 mutations were not identified, all had similar phenotypes (Tables 1 and 2). In addition, the increased variability of the phenotype in females which ranged from isolated β_2 microglobulinuria to the additional presence of hypercalciuria and nephrocalcinosis (such as patient 1 in Table 1) has been previously observed [10, 12, 13, 21] and is likely to result from the variability of X chromosome inactivation observed in females [13, 21]. The absence of CLCN5 mutations in families 9.2/95, 13.1/95, 20.2/95 and 25/95 (Table 1) may either be because these patients have another form of Dent's$_{JAPAN}$ or that

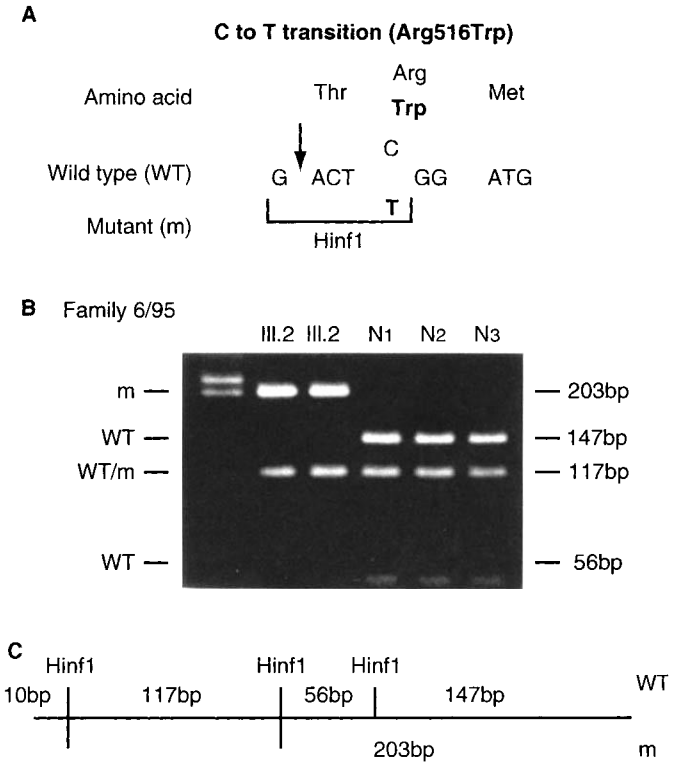

Fig. 3. Detection of mutation in exon 10 in family 6/95 by restriction enzyme analysis. DNA sequence analysis of the two affected male cousins III.2 and III.3 (patients 10 and 11 in Table 1, respectively) revealed a C to T transition at codon 516 (panel *A*), thus altering the wild-type (WT) sequence CGG, encoding an arginine (R) to the mutant (m) sequence, TGG, encoding a tryptophan (W). This missense mutation (R516W) also resulted in the loss of a *Hin*fI restriction enzyme site (G/ACTC). PCR amplification and *Hin*fI digestion (panel *B*) would result in four products of 147 bp, 117 bp, 56 bp and 10 bp (not shown) from the normal sequence but only three products of 203 bp, 117 bp and 10 bp (not shown) from the mutant sequence, as is illustrated in the restriction map in panel *C*. This R516W mutation was not present in 131 unaffected individuals [56 unrelated Japanese individuals (54 females, 2 males) and 75 (40 males, 35 females) unrelated Northern Europeans] N1 to N3 shown, thereby indicating that it is not a common DNA sequence polymorphism. The standard size marker in the form of the 1 Kb ladder is indicated. Similar restriction enzyme analysis was used to confirm the W279X and G513E mutations, and the T484T silent polymorphism (Table 2 and Fig. 2).

the mutation may be present in an as yet uncharacterized region such as the untranslated region of the 9.5Kb CLCN5 transcript [6] or the promoter. The mechanism(s) whereby CLCN5 mutations that cause a functional loss of CLC-5 and result in the low molecular weight proteinuria of the Dent's$_{JAPAN}$ phenotype (Table 1) remain to be defined. Low molecular weight proteins are specifically absorbed in the proximal tubule by endocytosis and transported in an acidic vacuolar-lysosomal system [24], and a loss of CLC-5 function in this system may prevent the dissipation of the charge that is generated by the electrogenic H^+-ATPase pump for the provision of the acidic environment [5, 6, 10]. This may lead to vesicle trafficking abnormalities with an initial failure of low molecular weight protein reabsorption and a subsequent failure of calcium reabsorption. Such possibilities remain to be explored and defining the intra-cellular location of CLC-5 in the human kidney will represent an important step in elucidating such possibilities.

The CLCN5 mutations appear to cluster in three regions. Firstly, 5 of the 10 mutations identified to date from the present and previous studies [5] of Dent's$_{JAPAN}$ families occur in exon 8 which encodes codons 269 to 449 [7]. Secondly, the W279X mutation, resulting from TGG→TGA, has been observed to occur in three unrelated families (2 Japanese and 1 British) [5, 10], and this represents a possible mutational hot-spot. Thirdly, transmembrane domain D11, which is highly conserved in other CLC members (> 95% identity between CLC-3, CLC-4 and CLC-5) [6] and is encoded by part of exon 10, has been observed to harbor 5 of the 26 reported mutations [5, 10, 20]. These results indicate that a possible useful strategy to investigate for CLCN5 mutations might be to first investigate exons 8 and 10 for mutations. Such mutational analysis may initially be undertaken by SSCP, which has detected over 80% of the 26 reported CLCN5 mutations (6 from the present study and 20 previously reported) [5, 10, 20]. Two of the Dent's$_{JAPAN}$ mutations (R347X and R516W) and three of the previously reported mutations (S244L, R648X and R704X) [10] involved a C to T transition that occurred at a CpG site, which is the most common site of methylation in mammalian DNA [22]. Such sites have been previously reported to represent potential "hot spots" for mutations as deamination of the 5-methylcytosine results in a C to T transition [23]. Our results revealing over 20% of CLCN5 mutations at such sites are consistent with these observations. In addition, our results indicate that CLCN5 mutations can be detected in over 70% of Dent's$_{JAPAN}$ patients and that over 80% of these mutations can be readily investigated for by SSCP. Thus, the results of our study will help in the genetic and clinical evaluation of patients at risk from Dent's$_{JAPAN}$ and other such similar X-linked renal proximal tubulopathies.

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