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Table 1 *CANT1* mutations in eight families with Desbuquois dysplasia

Patient ID	Subtype	Consanguinity	Ethnicity	Mutation	
				Paternal	Maternal
P1	1	No	Australian Caucasian	c.228_229insC (p.W77LfsX13)	c.671T→C (p.L224P)
P2*	2	Yes	Turkish	c.375G→C (p.W125C)	c.375G→C (p.W125C)
P3	2	Yes	Turkish	(-)	(-)
P4*	Kim variant	Yes	Japanese	c.676G→A (p.V226M)	c.676G→A (p.V226M)
P5	Kim variant	No	Japanese	c.861C→A (p.C287X)	c.676G→A (p.V226M)
P6	Kim variant	No	Japanese	c.676G→A (p.V226M)	c.494T→C (p.M165T)
P7	Kim variant	No	Korean	c.676G→A (p.V226M)	c.1079C→A (p.A360D)
P8*	Kim variant	No	Korean	c.676G→A (p.V226M)	IVS2-9G→A (p.G279VfsX8)

*There are two affected siblings in these families.

containing 4 mM CaCl₂, and 2 mM ADP or UDP, and incubated at 37°C for 1 min. The amounts of inorganic phosphate from ADP or UDP in the reaction were measured by a colorimetric molybdenum blue method.

Western blotting

Cell lysate was prepared using M-PER mammalian protein extraction reagents (Pierce, Rockford, IL, USA). Proteins in the cell lysate and the culture supernatant were separated by electrophoresis on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ, USA). The primary antibody to *CANT1* (Abcam, Cambridge, UK) was used at 1:1000 dilution, and then horseradish peroxidase conjugated anti-rabbit IgG (GE Healthcare, Chalfont St Giles, UK) was used at 1:2000 dilution. Chemiluminescent signals were detected using ECL plus western blotting detection reagents (Amersham).

RESULTS

Identification of *CANT1* mutations

We examined 11 subjects with DD from eight families (one type 1, two type 2, five Kim variant) and identified *CANT1* mutations in seven families, including all of those with DD Kim variant (table 1). Two homozygous mutations, c.375G→C (p.W125C) and c.676G→A (p.V226M), were found in two consanguineous families; others were compound heterozygous mutations. Co-segregation of mutations in the families was confirmed by sequencing genomic DNA of available family members. Altogether, we found eight distinct mutations; two were nonsense (p.W77LfsX13, p.C287X), five were missense (p.W125C, p.M165T, p.L224P, p.V226M, p.A360D), and one was at a splice acceptor site (IVS2-9G→A) (table 1, supplemental figure 1A). All mutations have not been reported so far.

To investigate the effect of IVS2-9G→A on splicing, we extracted RNA from peripheral leucocytes of patient 8. We cloned and sequenced its reverse transcriptase PCR (RT-PCR) products. We identified two distinct subclones: one had a 7 bp insertion between exons 2 and 3 (c.835_836insTTCCCAG) (supplemental figure 1B) and the other had c.676G→A (p.V226M). The former clone indicates that IVS2-9G→A generates a new splice acceptor site (supplemental figure 1C), which is predicted to produce a premature stop codon (p.G279VfsX8).

Among the eight mutations, c.676G→A was found in all families with Kim variant, and the other mutations were found only once. c.676G→A was found in 1/754 Japanese and 1/187 Korean controls in a heterozygous state. The other mutations were not found in approximately 100 ethnically matched controls, or in public sequence variation databases. No mutations were found in one type 2 family. We screened for *DTDST* mutations in this family as previously described,¹³ but found no mutation.

CANT1 belongs to the apyrase protein family.⁸⁻¹⁰ *CANT1* related apyrases have eight highly conserved regions, designated as nucleotidase conserved regions (NCRs) (figure 2).¹⁴ All amino acid residues substituted by the missense mutations in DD patients were located in NCRs and highly conserved among diverse species and related apyrases (figure 2).

Functional characterisation of *CANT1* missense mutations

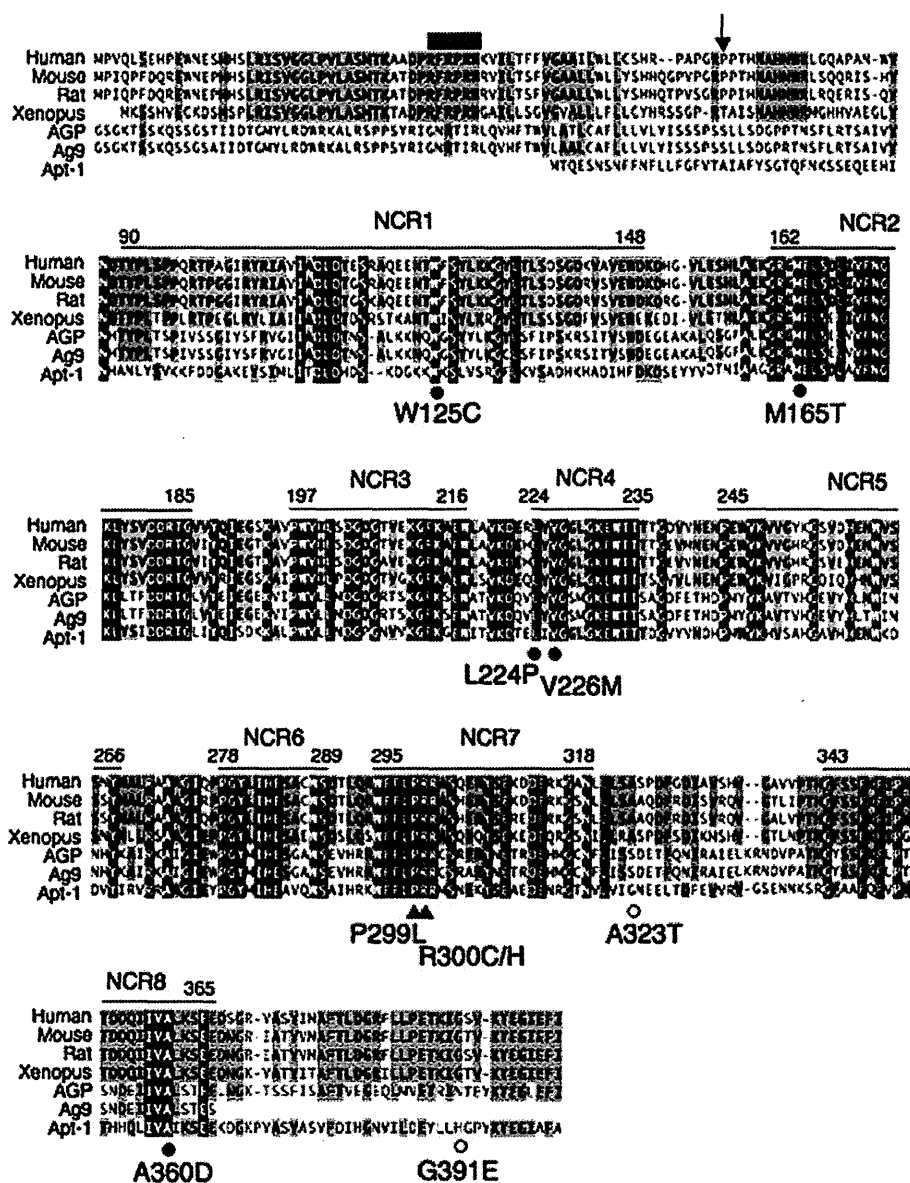
We evaluated the causality of the *CANT1* missense mutants by measuring their nucleotidase activity. We constructed expression vectors for the missense mutations identified in this study and the common mutation (p.R300C) previously identified,⁷ as well as two missense single nucleotide polymorphism (SNPs), p.A323T (rs9903215) and p.G391E (rs34082669) registered in dbSNP database (<http://www.ncbi.nlm.nih.gov/snp>) (figure 2). In our assay system, the wild-type *CANT1* was obviously calcium dependent and preferentially hydrolysed UDP (data not shown), as reported previously.⁸⁻⁹ Although *CANT1* hydrolyses ADP poorly, ADP hydrolysis by soluble apyrases has been reported to be involved in thrombo-regulation.¹⁰ Therefore, we measured nucleotidase activity of the mutants for both UDP and ADP. The activities of all DD mutant proteins were significantly reduced in both assays compared to that of the wild-type protein (figure 3A). The enzymatic activities of the SNP proteins were similar to that of the wild-type protein.

To investigate the stability and secretion of the missense mutant proteins, we checked the over-expressed proteins in cell lysates and culture supernatants by western blot analysis (figure 3B). The band intensities of four DD mutants (p.W125C, p.M165T, p.V226M, p.R300C) and two SNPs were equal to that of the wild-type in both analyses using cell lysates and culture supernatants, indicating that these proteins are stable and can be secreted normally into the culture supernatant. In contrast, the L224P band was drastically reduced in the cell lysate and not detectable in the culture supernatant, suggesting that L224P protein was unstable. An A360D band was at a similar level in the cell lysate but not detectable in the culture supernatant, indicating that A360D mutant could not be secreted into the culture medium. When the plasmid vector backbone was changed to another one, these results for the L224P and A360D mutants were unchanged.

DISCUSSION

We found eight novel *CANT1* mutations in seven of the eight DD families examined (type 1, type 2 and Kim variant). These included both nonsense and missense mutations, and our in vitro study showed the loss of *CANT1* enzyme activity in the missense mutants. Therefore, DD is caused by *CANT1* deficiency. Our study suggests that *CANT1* deficiency may be caused by early degradation and failure of secretion as well as the

Figure 2 Amino acid sequence alignment of human CANT1 and related apyrase proteins. Residues were shaded to indicate levels of conservation (black shape: complete conservation, grey shape: moderate conservation). Reference sequences: Human (AAH65038.1, *Homo sapiens* CANT1); Mouse (AH200mous03.1, *Mus musculus* CANT1); Rat (NP653355.1, *Rattus norvegicus* CANT1); *Xenopus* (AAH61377.1, *Xenopus tropicalis* CANT1); AGP (XP_321938.3, *Anopheles gambiae* apyrase); Ag9 (CAC35453, *A gambiae* apyrase); Apt-1 (NP_509283, *Caenorhabditis elegans* apyrase). NCR: nucleotidase conserved region. The numbers show the amino acid positions corresponding to human CANT1. The positions of the missense mutations identified in patients with Desbuquois dysplasia in this study and in a previous study and missense single nucleotide polymorphisms (SNPs) in the public database are indicated by solid circles, solid triangles and open circles, respectively. N-terminal RXR endoplasmic reticulum (ER) retention/retrieval motif of vertebrate CANT1 is represented by a black bar. Predicted signal peptide cleavage site is indicated by an arrow.



decreased enzyme activity secondary to a specific amino acid substitution. Further characterisation of the disease-causing mechanism of the missense mutations is necessary to gain insight into function and metabolism of the CANT1 protein.

The first study reported three distinct missense mutations.⁷ Two are recurrent mutations in R300 (R300C/H), and one in the neighbouring amino acid (P299); all are in NCR7. In our study, we found no mutation in this hot spot. All five missense mutations in this study were also in NCRs but were not clustered in a specific region. In contrast, we found a common mutation, V226M, in all five families with Kim variant. Although the previous paper stressed that R300 belongs to a pentad of alternating positively and negatively charged residues (D114, Q284, R300, Q365, and K394) that comprise a network of four salt bridges involved in the catalytic site of CANT1,⁷ the missense mutations identified in our study affected none of these residues.

The previous study examined only DD type 1 patients and identified CANT1 mutations.⁷ In this study, we identified CANT1 mutations in all types of DD, indicating that the

clinical–radiographic spectrum of CANT1 mutations must be extended to include distinct variants of Desbuquois syndrome. The V226M mutation was identified in all patients with DD Kim variant, suggesting that this mutation is necessary for the phenotype. However, because all patients with DD Kim variant in this study were East Asians, it may just reflect that V226M is prevalent among East Asians. Consistent with this hypothesis, the carrier frequency was indeed higher in this population. CANT1 mutation was not identified in one DD type 2 patient. Furthermore, linkage analysis excluded the possibility that CANT1 locus is responsible in three inbred DD families without typical hand abnormality (not included in the present study).⁶ Those results suggest that there may indeed be genetic heterogeneity in DD type 2. Further accumulation of the knowledge on phenotypes and mutations is required to gain a full picture of the phenotype–genotype association. In particular, diagnostic criteria for DD must now be revised with the knowledge offered by molecular definition. While presence or absence of an additional metacarpal bone has been used to distinguish subtypes of DD, this sign is not a distinctive criterion to predict the molecular basis in DD.

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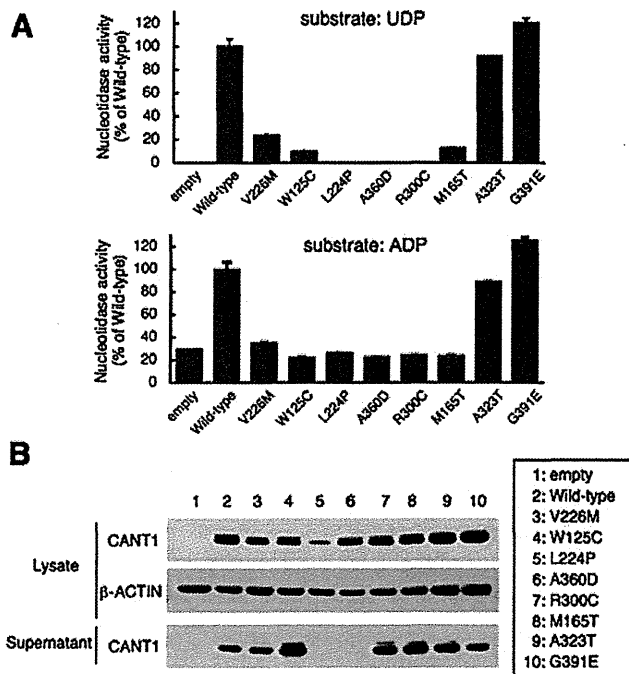


Figure 3 Functional characterisation of CANT1 missense mutants in Desbuquois dysplasia (DD) patients. (A) Nucleotidase activity of CANT1 mutants using uridine diphosphate (UDP) (upper panel) and adenosine diphosphate (ADP) (lower panel) as substrates. COS7 cells were transfected with expression vectors for empty, wild-type and missense mutant CANT1 proteins. The supernatant from the COS7 cells was used to measure the nucleotidase activity. W125C, M165T, L224P, V226M, R300C, A360D: missense mutants identified in DD; A323T, G391E: missense SNPs in the public database. Note that the nucleotidase activities of all DD missense mutants are significantly reduced compared to that of the wild-type protein in both assays. Results are presented as mean \pm SE (n=4). The same results were obtained from the independent experiments. (B) Western blot analysis for over-expressed CANT1 mutant proteins. Cell lysate and culture supernatant were prepared from COS-7 cells transfected with expression vectors for empty, wild type and missense mutant CANT1 proteins. The membranes with the cell lysates were stripped and re-probed with anti- β -actin antibody as a control. There were no bands for L224P and A360D in the supernatant. The same results were obtained from the independent experiments.

The exact function of CANT1 in humans remains unclear. CANT1 is a member of the apyrase family, which is classified into two groups based on amino acid sequence homology; one is the E-type ATPase family and the other is the family of apyrases cloned from haematophagous arthropods.^{10 15 16} CANT1 is classified in the latter group. Apyrases hydrolyse adenosine triphosphate (ATP) and ADP to adenosine monophosphate (AMP). ADP is one of the most important physiological agonists for platelet recruitment, aggregation and plug formation.¹⁷ Haematophagous insects secrete apyrases from their salivary gland to hydrolyse ADP, allowing them to feed on the host's blood for an extended time.¹⁸ The endothelial cell plasma membrane apyrase, CD39, an E-type ATPase, has also been implicated in thrombo-regulation.¹⁹ It has been shown that CANT1 has ADPase activity, but relatively low in comparison to its UDPase activity. An alternative hypothesis is that of CANT1 playing a role in making activated sugars available in the ER for synthesis of proteoglycans⁷ (see below). In this study, we again showed that CANT1 had ADPase activity and the missense mutants lost this activity (figure 3A). At present, the

physiological functions of CANT1 remain to be determined as does the possible role of thrombo-regulation in enchondral ossification and pathogenesis of DD.

Human CANT1 was cloned as a new member of extracellular nucleotidases⁸; however, mammalian CANT1 proteins have the N-terminus RXR, ER retention/retrial motif and that the over-expressed rat CANT1 preferentially localised to the ER.⁹ These findings suggest that CANT1 may exist as membrane bound forms in the ER as well as soluble forms. CANT1 substrates (UDP, GDP, UTP) are involved in several signalling functions including calcium (Ca^{2+}) release, through activation of pyrimidineric signalling.^{20–22} The binding of pyrimidineric nucleotides (UTP/UDP) to P2Y receptors generates inositol 1,4,5-triphosphate (IP_3) through their coupling to phospholipase C. IP_3 binding to its receptor at the ER surface causes rapid Ca^{2+} release from the ER stores.²¹ It has been reported that IP_3 receptor dependent Ca^{2+} release from the ER stores is increased during ER stress and plays a critical role in ER stress induced apoptosis.²³ Abnormally distended rough ER containing inclusion bodies was found in the chondrocytes and fibroblasts of DD patients.⁷ The abnormality may be related to impaired ER function caused by CANT1 mutations. The deletion of APY-1, the *Caenorhabditis elegans* homologue of CANT1, sensitised the worms to ER stress and induced defects in pharynx and muscle organisation, leading to a reduced lifespan.²⁴ Involvement of ER stress response in chondrogenesis and pathology of skeletal dysplasias has been reported.^{25 26}

DD shares some phenotypic features with diastrophic dysplasia (OMIM 222600) and recessive Larsen syndrome (OMIM 245600). Both are caused by deficiency of enzymes involved in the metabolism of chondroitin sulfate, an essential component of cartilage matrix. We have previously shown that a functional defect of the solute carrier-35 D1 (SLC35D1) caused a severe skeletal dysplasia in mouse and human.^{27 28} SLC35D1 is a nucleotide sugar transporter that transports UDP-N-acetylgalactosamine and UDP-glucuronic acid from the cytoplasm into the ER.^{29 30} The transported nucleotide sugars are utilised for synthesis of sugar chains of chondroitin sulfate.²⁷ The resulting UDP is hydrolysed to uridine monophosphate (UMP) by luminal nucleoside diphosphatase, and then UMP is exchanged via the antiporter system for importing further nucleotide sugars.³⁰ We speculate that CANT1 may work as this luminal nucleoside diphosphatase, thereby being involved in the nucleotide sugar/nucleoside monophosphate antiport system that is essential for cartilage development and functions. CANT1 deficiency might interfere with the availability of nucleotide sugars needed for chondroitin sulfate synthesis.

Key points

- ▶ CANT1 mutations were identified in seven out of eight Desbuquois dysplasia (DD) families. A total of eight distinct mutations were found; all were hitherto undescribed.
- ▶ Mutations were detected in DD type 2 and Kim variant. Thus, CANT1 is responsible for more than DD type 1.
- ▶ By measuring the CANT1 enzyme activity in vitro, we confirmed that missense mutations resulted in loss of function. Two missense mutations showed abnormal secretion.
- ▶ While the presence or absence of an additional metacarpal ossification centre has been used to distinguish subtypes of DD, this sign is not a distinctive criterion to predict the molecular basis in DD.

Our discovery has extended the phenotypic spectrum of *CANT1* mutations and shown that *CANT1* mutations are responsible not only for DD type 1, but also for type 2 and Kim variant. Further studies are necessary to characterise fully the role of *CANT1* in chondrogenesis and identify possible therapeutic targets. On the practical side, mutation analysis of *CANT1* may be warranted in all patients with a diagnostic suspicion of Desbuquois dysplasia, regardless of the specific hand phenotype.

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Competing interests None declared.

Patient consent Obtained.

Ethics approval This study was conducted with the approval of the Ethics Committee of RIKEN.

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