**CANT1 mutation is also responsible for Desbuquois dysplasia, type 2 and Kim variant**

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**ABSTRACT**

**Background** Desbuquois dysplasia (DD) is a recessively inherited condition characterised by short stature, generalised skeletal dysplasia and advanced bone maturation. DD is both clinically and radiographically heterogeneous, and two subtypes have been distinguished based on the presence (type 1) or absence (type 2) of an accessory metacarpal bone. In addition, an apparently distinct variant without additional metacarpal bone but with short metacarpals and long phalanges (Kim variant) has been described recently. Mutations in the gene that encodes for CANT1 (calcium-activated nucleotidase 1) have been identified in a subset of patients with DD type 1.

**Methods** A series of 11 subjects with DD from eight families (one type 1, two type 2, five Kim variant) were examined for CANT1 mutations by direct sequencing of all coding exons and their flanking introns.

**Results** Eight distinct mutations were identified in seven families (one type 1, one type 2 and all 5 Kim variant): three were nonsense and five were missense. All missense mutations occurred at highly conserved amino acids in the nucleotidase conserved regions of CANT1. Measurement of nucleotidase activity in vitro showed that the missense mutations were all associated with loss-of-function.

**Conclusion** The clinical-radiographic spectrum produced by CANT1 mutations must be extended to include DD type 2 and Kim variant. While 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.

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**INTRODUCTION**

Desbuquois dysplasia (DD; MIM 251450) is a severe skeletal dysplasia inherited in an autosomal recessive manner. DD belongs to the ‘multiple dislocation group’ in the International Nosology of Genetic Skeletal Disorders.1 It is characterised clinically by short limb short stature, severe joint laxity with facultative congenital dislocations, flat midface, micrognathia, cleft palate, and progressive scoliosis. The main radiological features include a peculiar ‘monkey wrench’ or ‘Swedish key’ appearance of the proximal femur (exaggerated trochanter), hypoplasia of thorax and ilia, mild spondylar dysplasia, and hand abnormalities including an additional ossification centre and advanced bone age.2

DD has been considered clinically and radiographically heterogeneous. It has been classified into two types on the basis of the presence (type 1) or absence (type 2) of characteristic hand anomalies, which consist of an extra ossification centre distal to the second metacarpal, delta phalanx, bifold distal thumb phalanx, and dislocation of the interphalangeal joints (figure 1A).3 DD type 2 is also referred to as the ‘normal’ hand type because the accessory metacarpal ossification centre is not seen. It only presents with minor changes of the hand, such as malalignment of the interphalangeal joint and brachydactyly. More than half of the DD patients belong to this subtype.3 In addition, we have recently described a new clinical subtype of DD, Kim variant.4 The hand shape of the variant is apparently normal, thus the patients most closely resemble type 2; however, the radiographic abnormalities in the hands are significant, including short metacarpals and elongated phalanges together with remarkably advanced carpal bone age (figure 1B). Long term follow-up showed that severe precocious osteoarthritis of the hand and spine is a major manifestation of this specific clinical variant.4

The apparent phenotypical heterogeneity of DD has been taken to suggest genetic heterogeneity. Thus, the DD gene was localised to a 1.65 Mb interval on chromosome 17q25 by homozygosy mapping using four consanguineous families with DD type 1, while DD with normal hands was believed not to map to this interval.5 6 Recently, Huber and colleagues searched for mutations in genes in the interval and identified mutations in the calcium activated nucleotidase 1 (CANT1) gene.7 CANT1 is a extracellular protein that functions as a nucleotide tri- and diphosphatase. It preferentially hydrolyses uridine diphosphate (UDP) followed by guanosine diphosphate (GDP), uridine triphosphate (UTP) and adenosine diphosphate (ADP).5–10

The function of CANT1 in skeletal formation is unknown. It is expressed in chondrocytes, and chondrocytes from DD type 1 patients with CANT1 mutations have abnormally distended rough endoplasmic reticulum (ER) implicating CANT1 in chondrocyte ER metabolism.7 The disease-causing mechanism of CANT1 mutation is also unclear. The first mutation study reported seven CANT1 mutations; three are nonsense mutations that are predicted to cause the nonsense mutation mediated RNA decay and one is a homoyzygous large deletion encompassing 5′-UTR and exon 1 that results in loss of CANT1 mRNA.7
These findings suggest that loss of CANT1 function causes DD. However, functional impact of the missense mutations has not been examined. It is of note that the genetic mapping was done using DD type 1 families, and that all patients in whom CANT1 mutations were identified had DD type 1. Therefore, it remains to be determined whether CANT1 is responsible for other types of DD.

To explore further the range of CANT1 mutations in DD, we searched for CANT1 mutations in three types (type 1, type 2, Kim variant) of DD patients. We found a total of eight distinct mutations in seven families (type 1, type 2, and all five Kim variant); all were novel. By measuring the CANT1 enzyme activity in vitro, we confirmed that DD results from CANT1 loss of function.

**PATIENTS AND METHODS**

**Patients**

DD patients were recruited through the International Skeletal Dysplasia Registry (ISDR) (http://www.csmc.edu/), the European Skeletal Dysplasia Network (ESDN) (http://www.esdn.org/), and the Japanese Skeletal Dysplasia Consortium (JSDC) (http://www.riken.jp/lab-www/OA-team/JSDC/). Clinical criteria for inclusion in the study were prenatal and postnatal growth failure with short limbs, mid-face hypoplasia or round face with flat nasal bridge, joint laxity and foot deformities. Major radiological criteria were Swedish key appearance of the proximal femur, advanced carpo-tarsal ossification, short tubular bones, and hyperphalangy (extra-ossicle between the proximal phalanx and metacarpal of the index finger) in type 1. Minor radiological criteria included hypoplastic lower ilia and vertebral modification, such as coronal clefts or irregular endplates at birth and mild vertebral flattening with round vertebral endplates or normal vertebral bodies in later life. Clinical and radiographic phenotypes of the patients were evaluated by the experts of the organisations and reviewed by the authors (SU, AS-F, OK, and GN).

Eleven patients with DD from eight families were recruited for this study (table 1). Clinical data of patient 1 have been reported previously (as patients 3, 5, 6, and 1, respectively). The study was approved by the ethics committee of RIKEN and participating institutions and informed consent was obtained from all subjects.

**Mutation screening**

Genomic DNA was extracted from blood by standard procedures or from saliva using Oragene DNA Self-Collection kit (DNA Genotek, Kanata Ontario, Canada). Exon sequences of CANT1 with their flanking intron sequences were amplified by PCR from genomic DNA. PCR primer sequences and PCR condition are listed in supplemental table 1. PCR products were purified using MinElute PCR purification Kit (Qiagen, Valencia, CA, USA) and sequenced for both strands using an ABI Prism 3730 automated sequencer (PE Biosystems, Foster City, CA, USA). Genomic DNAs from the parents and sibs were sequenced for the corresponding regions.

**cDNA cloning and in vitro mutagenesis**

The full length CANT1 cDNA was amplified by PCR using cDNAs prepared from OUMS-27 cells as a template and cloned into pcDNA3.1 (+). The missense mutations were introduced into CANT1 cDNA by PCR based mutagenesis. The introduced mutations were confirmed by DNA sequencing.

**Nucleotidase assay**

COS-7 cells were grown to 70–80% confluence in 100 mm culture dishes and were transfected with 6 μg of empty, wild-type or mutant CANT1 expression vectors using Fugene 6 transfection reagent (Roche Diagnostics, Basel, Switzerland). At 8 h after transfection, cells were transferred to Opti-MEM serum-free media and cultured for 48 h. Buffer exchange in the culture supernatant (10 ml) was performed with ultrafiltration through Amicon Ultra-10K centrifugal filter (Millipore, Billerica, MA, USA) using 50 ml of sterile deionised water and the resulting solution concentrated to 100 ml using VIVASPIN 500, 10000 MWCO (Vivascience, Hanover, Germany). Nucleotidase activities in the purified media were determined by modification of a previous method. Briefly, the purified media were 20 times or 500 times diluted with 40 mM succinate buffer (pH 6.5)
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.W125C, p.A360D), and two were missense (p.W125C, p.G391E). 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 mutations 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.A52ST (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.10,11 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 2A). 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 2B). The band intensities of four DD mutants (p.W125C, p.A360D, 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
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.
The same results were obtained from the independent experiments. There were no bands for L224P and A360D in the supernatant. missense mutants lost this activity (again showed that CANT1 had ADPase activity and the cloned from haematophagous arthropods. CANT1 is the E-type ATPase family and the other is the family of apyrases into two groups based on amino acid sequence homology; one is for platelet recruitment, aggregation and plug formation. (AMP). ADP is one of the most important physiological agonists triphosphate (ATP) and ADP to adenosine monophosphate for synthesis of proteoglycans (see below). In this study, we mean to that of the wild-type protein in both assays. Results are presented as mean±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 supernatant from the COS-7 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±SE (n=4). The same results were obtained from the independent experiments.  

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±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. 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. Haematophagous insects secrete apyrases from their salivary gland to hydrolyse ADP, allowing them to feed on the host’s blood for an extended time. The endothelial cell plasma membrane apyrase, CD59, an E-type ATPase, has also been implicated in thrombo-regulation. 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 proteoglycans (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 nucleotidases; however, mammalian CANT1 proteins have the N-terminus RXR, ER retention/retial motif and that the over-expressed rat CANT1 preferentially localised to the ER. 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 (Ca2+) release, through activation of pyrimidinergic signalling. The binding of pyrimidinergic nucleotides (UTP/UDP) to P2Y receptors generates inositol 1,4,5-triphosphate (IP3) through their coupling to phospholipase C. IP3 binding to its receptor at the ER surface causes rapid Ca2+ release from the ER stores. It has been reported that IP3 receptor dependent Ca2+ release from the ER stores is increased during ER stress and plays a critical role in ER stress induced apoptosis. Abnormally distended rough ER containing inclusion bodies was found in the chondrocytes and fibroblasts of DD patients. 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. Involvement of ER stress response in chondrogenesis and pathology of skeletal dysplasias has been reported.  

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. SLC35D1 is a nucleotide sugar transporter that transports UDP-N-acetylgalactosamine and UDP-glucronic acid from the cytoplasm into the ER. The transported nucleotide sugars are utilised for synthesis of sugar chains of chondroitin sulfate. 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. 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.

Team consensus Obtained.

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