for the gastrula organizer and its derivatives. BMC Genomics *9*, 511.

22. Suzuki, D., Yamada, A., Amano, T., Yasuhara, R., Kimura, A., Sakahara, M., Tsumaki, N., Takeda, S., Tamura, M., Nakamura, M., et al. (2009). Essential mesenchymal role of small GTPase Rac1 in interdigital programmed cell death during limb development. Dev. Biol. *335*, 396–406.
23. Kimura, S., and Shiota, K. (1996). Sequential changes of programmed cell death in developing fetal mouse limbs and its possible roles in limb morphogenesis. J. Morphol. *229*, 337–346.
24. Sernagor, E., Eglen, S.J., and Wong, R.O. (2001). Development of retinal ganglion cell structure and function. Prog. Retin. Eye Res. *20*, 139–174.
25. Bandyopadhyay, A., Tsuji, K., Cox, K., Harfe, B.D., Rosen, V., and Tabin, C.J. (2006). Genetic analysis of the roles of BMP2, BMP4, and BMP7 in limb patterning and skeletogenesis. PLoS Genet. *2*, e216.
26. Robert, B. (2007). Bone morphogenetic protein signaling in limb outgrowth and patterning. Dev. Growth Differ. *49*, 455–468.
27. Dudley, A.T., Lyons, K.M., and Robertson, E.J. (1995). A requirement for bone morphogenetic protein-7 during development of the mammalian kidney and eye. Genes Dev. *9*, 2795–2807.
28. Khokha, M.K., Hsu, D., Brunet, L.J., Dionne, M.S., and Harland, R.M. (2003). Gremlin is the BMP antagonist required for maintenance of Shh and Fgf signals during limb patterning. Nat. Genet. *34*, 303–307.
29. Furuta, Y., and Hogan, B.L. (1998). BMP4 is essential for lens induction in the mouse embryo. Genes Dev. *12*, 3764–3775.
30. Asai-Coakwell, M., French, C.R., Berry, K.M., Ye, M., Koss, R., Somerville, M., Mueller, R., van Heyningen, V., Waskiewicz, A.J., and Lehmann, O.J. (2007). GDF6, a novel locus for a spectrum of ocular developmental anomalies. Am. J. Hum. Genet. *80*, 306–315.
31. Tassabehji, M., Fang, Z.M., Hilton, E.N., McGaughran, J., Zhao, Z., de Bock, C.E., Howard, E., Malass, M., Donnai, D., Diwan, A., et al. (2008). Mutations in GDF6 are associated with vertebral segmentation defects in Klippel-Feil syndrome. Hum. Mutat. *29*, 1017–1027.
32. Wyatt, A.W., Osborne, R.J., Stewart, H., and Ragge, N.K. (2010). Bone morphogenetic protein 7 (BMP7) mutations are associated with variable ocular, brain, ear, palate, and skeletal anomalies. Hum. Mutat. *31*, 781–787.
33. Ye, M., Berry-Wynne, K.M., Asai-Coakwell, M., Sundaresan, P., Footz, T., French, C.R., Abitbol, M., Fleisch, V.C., Corbett, N., Allison, W.T., et al. (2010). Mutation of the bone morphogenetic protein GDF3 causes ocular and skeletal anomalies. Hum. Mol. Genet. *19*, 287–298.
34. Zhou, F., Leder, P., Zuniga, A., and Dettenhofer, M. (2009). Formin1 disruption confers oligodactylism and alters Bmp signaling. Hum. Mol. Genet. *18*, 2472–2482.
35. Chang, B., Smith, R.S., Peters, M., Savinova, O.V., Hawes, N.L., Zabaleta, A., Nusinowitz, S., Martin, J.E., Davisson, M.L., Cepko, C.L., et al. (2001). Haploinsufficient Bmp4 ocular phenotypes include anterior segment dysgenesis with elevated intraocular pressure. BMC Genet. *2*, 18.
36. Ragge, N.K., Brown, A.G., Poloschek, C.M., Lorenz, B., Henderson, R.A., Clarke, M.P., Russell-Eggitt, I., Fielder, A., Gerrelli, D., Martinez-Barbera, J.P., et al. (2005). Heterozygous mutations of OTX2 cause severe ocular malformations. Am. J. Hum. Genet. *76*, 1008–1022.
37. Behesti, H., Papaioannou, V.E., and Sowden, J.C. (2009). Loss of Tbx2 delays optic vesicle invagination leading to small optic cups. Dev. Biol. *333*, 360–372.

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

Tatsuya Furuichi,[1,2] Jin Dai,[1] Tae-Joon Cho,[3] Satoru Sakazume,[4] Masahide Ikema,[5] Yoshito Matsui,[6] Gareth Baynam,[7] Toshiro Nagai,[4] Noriko Miyake,[8] Naomichi Matsumoto,[8] Hirofumi Ohashi,[9] Sheila Unger,[10] Andrea Superti-Furga,[10] Ok-Hwa Kim,[11] Gen Nishimura,[12] Shiro Ikegawa[1]

► Additional figures and tables are published online only. To view these files please visit the journal online (http://jmg.bmj.com).

[1]Laboratory of Bone and Joint Diseases, Center for Genomic Medicine, RIKEN, Tokyo, Japan
[2]Laboratory Animal Facility, Research Center for Medical Sciences, Jikei University School of Medicine, Tokyo, Japan
[3]Department of Orthopaedic Surgery, Seoul National University Children's Hospital, Seoul, Korea
[4]Department of Pediatrics, Dokkyo Medical University Kosigaya Hospital, Koshigaya, Japan
[5]Department of Orthopaedic, Nagasaki Prefectural Center of Medicine and Welfare for Children, Nagasaki, Japan
[6]Department of Orthopedic Surgery, University of Toyama, Toyama, Japan
[7]Genetic Services of Western Australia, Princess Margaret Hospital for Children and King Edward Memorial Hospital for Women, Western Australia, Australia
[8]Department of Human Genetics, Yokohama City University Graduate School of Medicine, Yokohama, Japan
[9]Division of Medical Genetics, Saitama Children's Medical Center, Iwatsuki, Japan
[10]Center for Pediatrics and Adolescent Medicine, University of Freiburg, Freiburg, Germany
[11]Department of Radiology, Ajou University Hospital, Suwon, Korea
[12]Department of Pediatric Imaging, Tokyo Metropolitan Children's Medical Center, Futyu, Japan

Correspondence to
Dr Shiro Ikegawa, Laboratory of Bone and Joint Diseases, Center for Genomic Medicine, RIKEN, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan; sikegawa@ims.u-tokyo.ac.jp

Received 8 May 2010
Revised 30 June 2010
Accepted 3 July 2010
Published Online First 30 October 2010

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.

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, bifid 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 brachydactly. 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 homozygosity 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).[8–10]

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 homozygous large deletion encompassing 5′-UTR and exon 1 that results in loss of *CANT1* mRNA.[7]

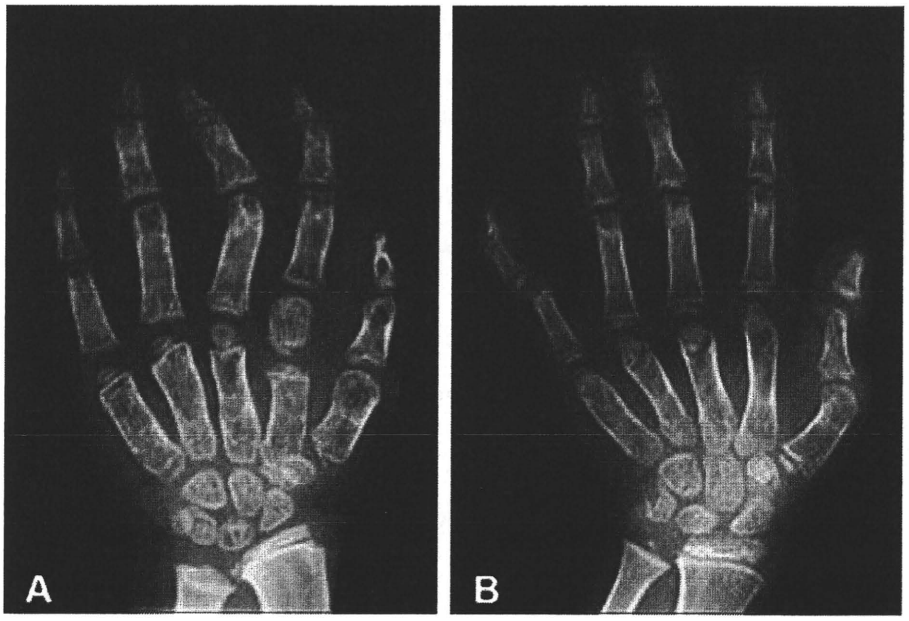

Figure 1 Hand radiographs of Desbuquois dysplasia (DD) type 1 and Kim variant at age 3 years. (A) DD type 1 carrying the homozygous *CANT1* mutation, p.R300C. This girl has been reported previously as family 4.[7] Note the additional ossification centre at the second metacarpal. (B) DD Kim variant (patient 7 in the present study) carrying the compound heterozygous *CANT1* mutations, p.V226M and p.A360D. There is no additional ossification centre. Both patients show precocious carpal ossification.

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.[7] 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.[11] Patients 4, 5, 7, and 8 have also been reported previously (as patients 3, 5, 6, and 1, respectively).[4] 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.[12] Briefly, the purified media were 20 times or 500 times diluted with 40 mM succinate buffer (pH 6.5)

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 $CaCl_2$, 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. Cosegregation 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 hydrolysation 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

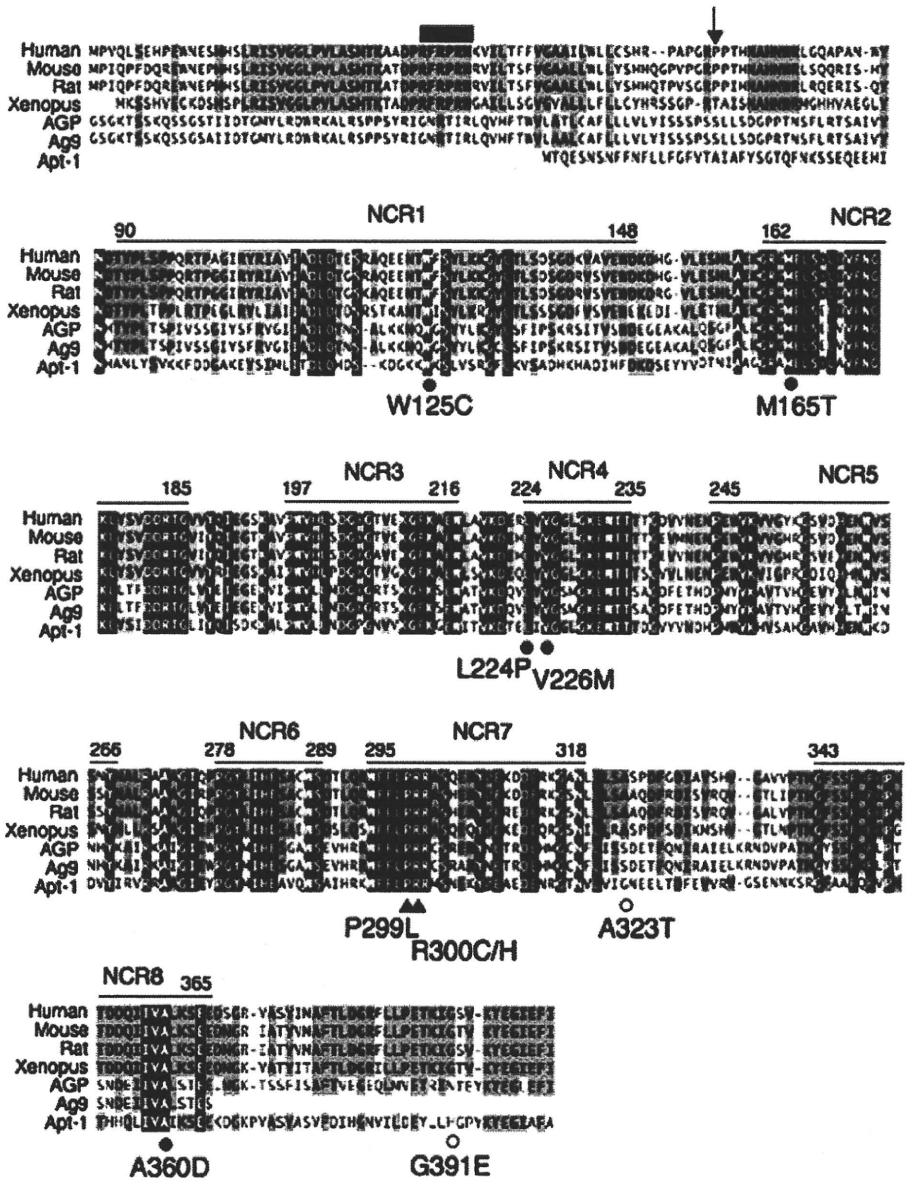

Figure 2 Amino acid sequence alignment of human CANT1 and related apyrase proteins. Residues were shaded to indicate levels of conservation (black shape: complete conservation, grey shape: moderate conservation). Reference sequences: Human (AAH65038.1, *Homo sapiens* CANT1); Mouse (AH200mous03.1, *Mus musculus* CANT1); Rat (NP653355.1, *Rattus norvergicus* CANT1); Xenopus (AAH61377.1, *Xenopus tropicalis* CANT1), AGP (XP_321938.3, *Anopheles gambiae* apyrase); Ag9 (CAC35453, *A gambiae* apyrase); Apt-1 (NP_509283, *Caenrohabditis 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.

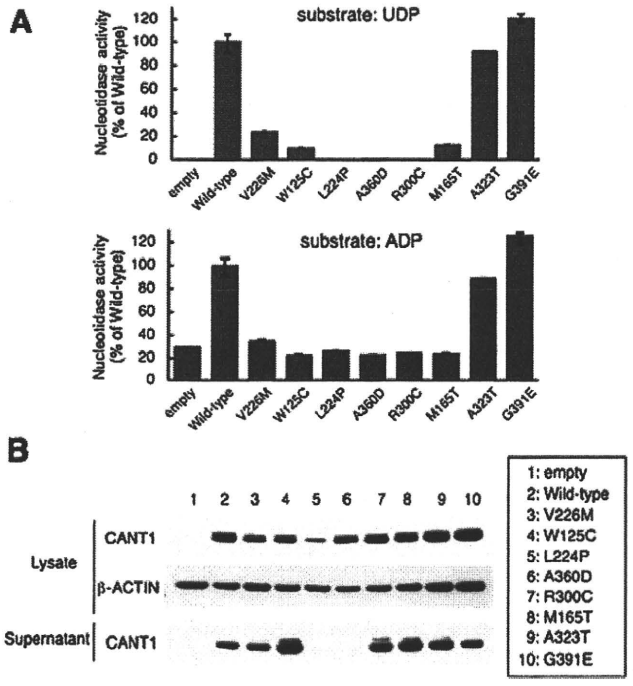

Figure 3 Functional characterisation of CANT1 missense mutants in Desbuquois dysplasia (DD) patients. (A) Nucleotidase activity of CANT1 mutants using uridine diphosphate (UDP) (upper panel) and adenosine diphosphate (ADP) (lower panel) as substrates. COS7 cells were transfected with expression vectors 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.[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 secret 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 apyarase, 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 proteoglycans[7] (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[8]; 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 pyrimidinergic signalling.[20–22] The binding of pyrimidinergic 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 IP3 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 *Caenrohabditis 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-glucronic 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.

J Med Genet 2011;**48**:32–37. doi:10.1136/jmg.2010.080226

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.

Acknowledgements We are grateful to Dr S Tominaga for her help in this study. We are also grateful to the patients, their family members, and their doctors for participating in the study. This project was supported by grants-in-aids from the Ministry of Education, Culture, Sports and Science of Japan (Contract grant No. 20390408 and No. 21249024), from Research on Child Health and Development (Contract grant No. 20-S-3), from the Ministry of Health, Labour and Welfare of Japan (Measures for Intractable Diseases 046 in 2010), from the Korea Healthcare Technology R&D Project, Ministry for Health, Welfare and Family Affairs, Republic of Korea (No. A080588), from the German Ministry for Education and Research (BMBF contract grant "SKELNET"), from the European Union (FP6, Contract grant "EuroGrow") and from the University of Freiburg. EL and BZ are supported by individual grants from Deutsche Forschungsgemeinschaft (La 1381/1-3). This project was also supported by the European Skeletal Dysplasia Network ESDN (http://www.esdn.org), The University of Freiburg, and the Japanese Skeletal Dysplasia Consortium (http://www.riken.jp/lab-www/OA-team/JSDC/).

Competing interests None declared.

Patient consent Obtained.

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

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES

1. **Superti-Furga A,** Unger S. Nosology and classification of genetic skeletal disorders: 2006 revision. *Am J Med Genet A* 2007;**143**:1–18.
2. **Desbuquois G,** Grenier B, Michel J, Rossignol C. Nanisme chondrodystrophique avec ossification anarchique et polymalformations chez deux soeurs. *Arch Fr Pediatr* 1966;**23**:573–87.
3. **Faivre L,** Cormier-Daire V, Eliott AM, Field F, Munnich A, Maroteaux P, Le Merrer M, Lachman R. Desbuquois dysplasia, a reevaluation with abnormal and "normal" hands: radiographic manifestations. *Am J Med Genet A* 2004;**124**:48–53.
4. **Kim OH,** Nishimura G, Song HR, Matsui Y, Sakazume S, Yamada M, Narumi Y, Alanay Y, Unger S, Cho TJ, Park SS, Ikegawa S, Meinecke P, Superti-Furga A. A variant of Desbuquois dysplasia characterized by advanced carpal bone age, short metacarpals, and elongated phalanges: report of seven cases. *Am J Med Genet A* 2010;**152**:875–85.
5. **Faivre L,** Le Merrer M, Al-Gazali LI, Ausems MGEM, Bitoun P, Bacq D, Maroteaux P, Munnich A, Cormier-Daire V. Homozygosity mapping of a Desbuquois dysplasia locus to chromosome 17q25.3. *J Med Genet* 2003;**40**:282–4.
6. **Faivre L,** Le Merrer M, Zerres K, Ben Hariz M, Scheffer D, Young ID, Maroteaux P, Munnich A, Cormier-Daire V. Clinical and genetic heterogeneity in Desbuquois dysplasia. *Am J Med Genet A* 2004;**128**:29–32.
7. **Huber C,** Oules B, Bertoli M, Chami M, Fradin M, Alanay Y, Al-Gazali LI, Ausems MGEM, Bitoun P, Cavalcanti DP, Krebs A, Le Merrer M, Mortier G, Shafeghati Y, Superti-Furga A, Robertson SP, Le Goff C, Muda AO, Paterlini-Brechot P, Munnich A, Cormier-Daire V. Identification of *CANT1* mutations in Desbuquois dysplasia. *Am J Hum Genet* 2009;**85**:706–10.
8. **Smith TM,** Hicks-Berger CA, Kim S, Kirley TL. Cloning, expression, and characterization of a soluble calcium-activated nucleotidase, a human enzyme belonging to a new family of extracellular nucleotidases. *Arch Biochem Biophys* 2002;**406**:105–15.
9. **Failer BU,** Braun N, Zimmermann H. Cloning, expression, and functional characterization of a Ca^{2+}-dependent endoplasmic reticulum nucleoside diphosphatase. *J Bio Chem* 2002;**40**:36978–86.
10. **Smith TM,** Kirley TL. The calcium activated nucleotidases: a diverse family of soluble and membrane associated nucleotide hydrolyzing enzymes. *Purinergic Signal* 2006;**2**:327–33.
11. **Baynam G,** Kiraly-Borri C, Goldblatt J, Dickinson JE, Jevon GP, Overkov A. A recurrence of a hydrop lethal skeletal dysplasia showing similarity to Desbuquois dysplasia and a proposed new sign: the Upsilon sign. *Am J Med Genet A* 2010;**152**:966–9.
12. **Traverso-cori A,** Chaimovich H, Cori O. Kinetic studies and properties of potato apyrase. *Arch Biochem Biophys* 1965;**109**:173–84.
13. **Maeda K,** Miyamoto Y, Sawai H, Karniski LP, Nakashima E, Nishimura G, Ikegawa S. A compound heterozygote harboring novel and recurrent *DTDST* mutations with intermediate phenotype between atelosteogenesis type II and diastrophic dysplasia. *Am J Med Genet A* 2006;**140**:1143–7.
14. **Yang M,** Kirley TL. Site-directed mutagenesis of human soluble calcium-activated nucleotidase 1 (hSCAN-1): identification of residues essential for enzyme activity and the Ca^{2+}-induced conformational change. *Biochemistry* 2004;**43**:9185–94.
15. **Plesner L.** Ecto-ATPases: identification and functions. *Int Rev Cytol* 1995;**158**:141–214.
16. **Valenzuela JG,** Charlab R, Galperin MY, Ribeiro JM. Purification, cloning, and expression of an apyrase from the bed bug cimex lectularis. A new type of nucleotide-bindind enzyme. *J Biol Chem* 1998;**273**:30583–90.
17. **Benoit P,** Dogne JM. Platelet ADP-receptors and their antagonists. *Mini Rev Med Chem* 2003;**3**:145–8.
18. **Ribeiro JM,** Francischetti IM. Role of arthropod saliva in blood feeding: sialome and post-sialome perspectives. *Annu Rev Entomol* 2003;**48**:73–88.
19. **Marcus AJ,** Broekman MJ, Drosopoulos JH, Islam N, Alyonycheva TN, Safier LB, Hajjar KA, Posnett DN, Schoenborn MA, Schooley KA, Gayle RB, Maliszewski CR. The endothelial cell ecto-ADPase responsible for inhibition of platelet function is CD39. *J Clin Invest* 1997;**99**:1351–60.
20. **Abbracchio MP,** Burnstock G, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, Knight GE, Fumagalli M, Gachet C, Jacobson KA, Weisman GA. International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. *Pharmacol Rev* 2006;**58**:281–341.
21. **Clapham DE.** Calcium signaling. *Cell* 2007;**131**:1047–58.
22. **Lecca D,** Ceruti S. Uracil nucleotides: from metabolic intermediates to neuroprotection and neuroinflammation. *Biochem Pharmacol* 2008;**75**:1869–81.
23. **Li G,** Mongillo M, Chin KT, Harding H, Ron D, Marks AR, Tabas I. Role of ERO1-alpha-mediated stimulation of inositol 1,4,5-triphosphate receptor activity in endoplasmic reticulum stress-induced apoptosis. *J Cell Biol* 2009;**186**:783–92.
24. **Uccelletti D,** Pascoli A, Farina F, Alberti A, Mancini P, Hirschberg CB, Palleschi C. APY-1, a novel Caenorhabditis elegans apyrase involved in unfolded protein response signalling and stress responses. *Mol Biol Cell* 2008;**19**:1337–45.
25. **Saito A,** Hino S, Murakami T, Kanemoto S, Kondo S, Saitoh M, Nishimura R, Yoneda T, Furuichi T, Ikegawa S, Ikawa M, Okabe M, Imaizumi K. Regulation of endoplasmic reticulum stress response by a BBF2H7-mediated Sec23a pathway is essential for chondrogenesis. *Nat Cell Biol* 2009;**11**:1197–204.
26. **Rajpar MH,** McDermott B, Kung L, Eardley R, Knowles L, Heeran M, Thornton DJ, Wilson R, Bateman JF, Poulsom R, Arvan P, Kadler KE, Briggs MD, Boot-Handford RP. Targeted induction of endoplasmic reticulum stress induces cartilage pathology. *PLoS Genet* 2009;**5**:e1000691.
27. **Hiraoka S,** Furuichi T, Nishimura G, Shibata S, Yanagishita M, Rimoin DL, Superti-Furga A, Nikkels PG, Ogawa M, Katsuyama K, Toyoda H, Kinoshita-Toyoda A, Ishida N, Isono K, Sanai Y, Cohn DH, Koseki H, Ikegawa S. Nucleotide-sugar transporter SLC35D1 is critical to chondroitin sulfate synthesis in cartilage and skeletal development in mouse and human. *Nat Med* 2007;**13**:1363–7.
28. **Furuichi T,** Kayserili H, Hiraoka S, Nishimura G, Ohashi H, Aslanger AD, Koseki H, Cohn DH, Superti-Furga A, Unger S, Ikegawa S. Identification of loss-of-function mutations of *SLC35D1* in patients with Schneckenbecken dysplasia, but not with other severe spondylodysplastic dysplasias group diseases. *J Med Genet* 2009;**46**:562–8.
29. **Muraoka M,** Kawakita M, Ishida N. Molecular characterization of human UDP-glucuronic acid/UDP-N-acetylgalactosamine transporter, a novel nucleotide sugar transporter with dual substrate specificity. *FEBS Lett* 2001;**495**:87–93.
30. **Ishida N,** Kawakita M. Molecular physiology and pathology of the nucleotide sugar transporter family (SLC35). *Pflugers Arch* 2004;**447**:768–75.