ARTICLE

A novel missense mutation in ANO5/TMEM16E is causative for gnathodiaphyseal dysplasia in a large Italian pedigree

Caterina Marconi¹, Paolo Brunamonti Binello², Giovanni Badiali³, Emanuela Caci⁴, Roberto Cusano^{5,6}, Joseph Garibaldi⁷, Tommaso Pippucci¹, Alberto Merlini², Claudio Marchetti³, Kerry J Rhoden¹, Luis JV Galletta⁴, Faustina Lalatta⁸, Paolo Balbi² and Marco Seri^{*,1}

Gnathodiaphyseal dysplasia (GDD) is an autosomal dominant syndrome characterized by frequent bone fractures at a young age, bowing of tubular bones and cemento-osseous lesions of the jawbones. Anoctamin 5 (ANO5) belongs to the anoctamin protein family that includes calcium-activated chloride channels. However, recent data together with our own experiments reported here add weight to the hypothesis that ANO5 may not function as calcium-activated chloride channel. By sequencing the entire ANO5 gene coding region and untranslated regions in a large Italian GDD family, we found a novel missense mutation causing the p.Thr513Ile substitution. The mutation segregates with the disease in the family and has never been described in any database as a polymorphism. To date, only two mutations on the same cysteine residue at position 356 of ANO5 amino-acid sequence have been described in GDD families. As ANO5 has also been found to be mutated in two different forms of muscular dystrophy, the finding of this third mutation in GDD adds clues to the role of ANO5 in these disorders.

European Journal of Human Genetics (2013) **21**, 613–619; doi:10.1038/ejhg.2012.224; published online 10 October 2012

Keywords: gnathodiaphyseal dysplasia; jawbone disease; anoctamin 5; calcium-activated chloride channel

INTRODUCTION

Gnathodiaphyseal dysplasia (GDD) is an autosomal dominant generalized skeletal syndrome characterized by cemento-osseous lesions of the jawbones, in conjunction with bone fragility, bowing/cortical thickening of tubular bones and diaphyseal sclerosis of long bones.¹

GDD was first described by Akasaka *et al*² in a Japanese four-generation family including 21 patients, exhibiting frequent bone fractures at a young age, purulent osteomyelitis of the jaws during adult life and an autosomal dominant inheritance. Riminucci *et al*³ studied the disease in depth from a clinical and histological point of view and proposed the name 'gnathodiaphyseal dysplasia', describing it as an autosomal dominant syndrome characterized by fibro-osseous lesions of the jawbones with a prominent psammomatoid body component that produces facial deformity, fractures due to bone fragility and characterized by normal healing without bone deformity, and bowing/sclerosis of tubular bones.

For years, the molecular and genetic basis of this pathological entity remained unclear. In 2004, Tsutsumi *et al*⁴ discovered a novel *GDD1* gene, also known as *TMEM16E* or anoctamin 5 (*ANO5*), which is mutated in the original Japanese family and also in an African-American family.

The human *ANO5* gene maps to chromosome 11p14.3-15.1 and encodes a 913 amino-acid protein whose molecular function and

biochemical properties have not yet been characterized. *ANO5* was found to be a member of the novel *TMEM16* gene family.⁵ Members of this gene family have a common structural topology with eight transmembrane domains, a re-entrant loop between the fifth and sixth transmembrane domain forming the channel pore,⁶ and a unique sequence motif called the annotated domain of unknown function 590 (DUF590). Several reports have demonstrated that *ANO1*(*TMEM16A*) and *ANO2*(*TMEM16B*) encode Ca²⁺-activated Cl⁻ channels^{6–10} and until recently it was supposed that all members of this family shared the same function. In 2010, Schreiber *et al*¹¹ showed that some but not all anoctamins are able to produce Ca²⁺-dependent Cl⁻ currents. The lack of Cl⁻ channel activity for some anoctamins, particularly for *ANO3* to *ANO7* (*TMEM16C* to *TMEM16G*), may be due to an intracellular localization as reported by Duran *et al*.¹² In this respect, Milenkovic *et al*¹³ showed that different members of this gene-family may have evolved different functional properties following duplication of a common ancestor and replacement of critical amino acids. Interestingly, *ANO6* (*TMEM16F*) was found to be an essential component of the widely distributed outwardly rectifying chloride channels,¹⁴ and also to be necessary for the Ca²⁺-dependent exposure of phosphatidylserine on the plasma membrane of cells, a process involved in blood coagulation and apoptosis.¹⁵

¹Unità di Genetica Medica, Dipartimento di Scienze Ginecologiche, Ostetriche e Pediatriche, Università di Bologna, Bologna, Italy; ²Struttura Complessa di Odontostomatologia, Dipartimento di Chirurgie Specialistiche, Ospedale Galliera, Genova, Italy; ³Unità di Chirurgia Orale e Maxillo-facciale, Dipartimento di Scienze Odontostomatologiche, Università di Bologna, Bologna, Italy; ⁴Laboratorio di Genetica Molecolare, Istituto Giannina Gaslini, Genova, Italy; ⁵Istituto di Ricerca Genetica e Biomedica-CNR, Monserrato, Italy; ⁶CRS4, Laboratorio di Genomica, Parco tecnologico della Sardegna, Pula Cagliari, Italy; ⁷Odontoiatra, Genova, Italy; ⁸Unità di Genetica Medica, Clinica Mangiagalli, Dipartimento area salute della Donna, del Bambino e del Neonato Fondazione IRCCS Cà Granda, Ospedale Maggiore Policlinico, Milano, Italy

*Correspondence: Professor M Seri, Medical Genetics Unit, Department of Gynaecological, Obstetric and Paediatric Sciences, University of Bologna, Via Massarenti 9, Bologna 40138, Italy. Tel: +39 051 208 8421; Fax: +39 051 208 8416; E-mail: marco.seri@unibo.it

Received 17 April 2012; revised 6 September 2012; accepted 6 September 2012; published online 10 October 2012

According to Tsutsumi *et al*¹⁶ and Mizuta *et al*¹⁷, ANO5 protein is suspected to be an integral membrane glycoprotein, residing predominantly within intracellular membrane vesicles and also in the plasma membrane, with a role in myogenesis and osteogenesis. In human tissues, ANO5 mRNA is detected in the brain, heart, kidney, lung and skeletal muscle, and in mice shows abundant expression in bone tissues including the calvarium, femur and mandible.⁴

The recent finding of recessive mutations in ANO5 in patients with proximal LGMD2L (limb-girdle muscular dystrophy-2l) and distal MMD3 (miyoshi muscular dystrophy-3) muscular dystrophies^{18,19} complicates the challenge of unraveling the molecular pathophysiology of GDD, which is still unclear.

In this work, we report the third mutation occurring in the ANO5 gene within a large Italian family affected by GDD, as well as functional studies indicating that ANO5, in contrast to ANO1 and ANO2, does not work as a plasma membrane Ca^{2+} -activated Cl^{-} channel.

MATERIALS AND METHODS

Clinical and laboratory evaluation

The family is composed of 55 subjects, 16 of them were affected (Figure 1).

Two affected subjects, V1 and V5, were available for direct clinical evaluation. Radiological exams of the legs and jawbones were performed on both V1 and V5, and bone scintigraphy on V5. Both patients underwent surgery of the jaw and mandible, and material from this intervention was submitted for histological and ultrastructural analyses. Hematological exams were carried out on patient V5.

Accurate clinical information was available for four other affected members and is detailed in Table 1.

Mutational screening

Mutational analysis was performed by PCR amplification of all coding exons, intron–exon junctions and 5' untranslated region of the ANO5 gene, followed by bidirectional sequencing of PCR fragments using the Bigdye kit v1.1 (Life Technologies, Carlsbad, CA, USA). Primers details are available upon request. The reference sequences are NC_000011.9 and NM_213599.2. Screening was carried out on genomic DNA extracted from peripheral blood, available from 21 individuals of the family (7 affected, 14 not affected) (Figure 1).

Cloning of ANO5 coding sequence

We evaluated different cell types looking for expression of ANO5 at the mRNA level. For this purpose, we used real-time quantitative PCR using inventoried Assays-on-Demand provided by Life Technologies (Hs01381106_m1 for ANO5 gene and Hs00187842_m1 for the gene encoding β 2-microglobulin, used as a reference gene). After this analysis, we found that the osteosarcoma cell line Saos-2 has a significant ANO5 expression. To clone the full-length ANO5 coding sequence, Saos-2 (cultured in McCoy's 5a medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin) were used to extract total RNA with Trizol (Gibco–BRL, Life Technologies, Carlsbad, CA, USA) and RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instruction. One microgram of spectrophotometer-quantified RNA was retrotranscribed using Advantage RT-for-PCR kit (BD-Clontech, Mountain View, CA, USA). Amplification of full-length human ANO5 coding sequence was obtained through PCR. The amplification product (2 μ l) was cloned in the pcDNA3.1 TOPO vector (Life Technologies) following the manufacturer's instructions. To identify the constructs with the correct orientation of the insert, PCR was performed from bacterial colonies.

Cell transfection

HEK-293 cells were seeded in 96-well microplates (25 000 cells/well) in 100 μ l of antibiotic-free culture medium (DMEM/Ham's F12 supplemented with 10% fetal calf serum). After 6 h, cells were co-transfected with plasmids carrying the coding sequence for ANO5, ANO1 and ANO2 proteins plus the halide-sensitive yellow fluorescent protein YFP-H148Q/I152L.²⁰ For each well, 0.2 μ g of total plasmid DNA and 0.5 μ l of Lipofectamine 2000 (Life Technologies) were first pre-mixed in 50 μ l of OPTI-MEM (Life Technologies) to generate transfection complexes (60 min at room temperature), and then added to the cells. After 24 h, the complexes were removed by replacement with fresh culture medium plus antibiotics. The YFP-based functional assay was performed after further 24 h.

Functional assay

Transfected HEK-293 cells were washed twice with 100 μ l PBS and incubated for 30 min with 60 μ l PBS. After incubation, cells were transferred to a fluorescence microscope equipped with a 20 $\times$ objective, optical filters for detection of EYFP fluorescence (excitation: HQ500/20 $\times$, 500 $\pm$ 10 nm; emission: HQ535/30 M, 535 $\pm$ 15 nm; dichroic: 515 nm; Chroma, Rockingham, VT, USA) and a photomultiplier tube (Hamamatsu, Shizuoka, Japan). For each well, cell fluorescence was continuously measured before and after addition of 165 μ l of a modified PBS containing 137 mM KI instead of NaCl (final

Table 1 Clinical characteristics of six affected members

Patient	Age	Mutation <i>p.T513I</i>	Cemento-oss eous lesions of the jawbones	Other jawbones features	Fractured bones with slow healing	Age of first fracture	Thickening of dia physeal cortex of long tubular bones	Serum CPK ^a	Other bone findings
V1	42	Yes	Yes	Jawbones sclerosis, right jaw angle structural rearrangement, jaw odontoid cystis with included tooth purulent osteomyelitis	Vertebra (atlas/epistropheus), thighbone (twice), fibula, humerus, radius/ulna, III right metatarsus, right iliac wing, hallux	8	Yes	112 mU/ml	First sacral vertebra partial cleft, knees and shoulders arthrosis
V5	41	Yes	Yes	Bone thickening of both jaws with areas of cotton-wool-like pattern of the alveo- lar region	Both thighbones more than once	10	Yes	>809 mU/ml	Reduced bone density with severe porosis of the knee and tibial bones
IV5	76	Yes	Yes	Purulent osteomyelitis, bilateral osteo- lysis, jaw structural rearrangement	Right knee, V left finger	12	No	89 mU/ml	Gum formation: fibrous tissue with ossification areas
IV10	70	Yes	Yes	Purulent osteomyelitis	No	ND	ND	ND	None
IV15	75	Yes	Yes	Inferior arch sclerosis, bilateral period- ontopathy, bones thickening areas	Thighbones, humerus	50	No	132 mU/ml	Temporal skull hyperostosis, osteophytes of dorsal and lumbar vertebrae, polyarthrosis
V12	13	ND	No	ND	Thighbone, clavicle	11	No	ND	None

Abbreviation: ND, not determined.
^aNormal CPK range 35–145 mU/ml.

I⁻ concentration in the well: 100 mM) with or without ionomycin 1 μM. The output from the photomultiplier tube was digitized using a PowerLab 2/25 acquisition system (ADInstruments, Sydney, NSW, Australia). After background subtraction, cell fluorescence recordings were normalized for the initial average value measured before addition of I⁻. The signal decay caused by YFP fluorescence quenching was fitted with a double exponential function to derive the maximal quenching rate that corresponds to initial influx of I⁻ into the cells.

RESULTS

Clinical evaluation

The clinical history of subject V5 was characterized by multiple spontaneous fractures of both thighbones with slow healing, and starting at age 11. Radiological exams showed generalized reduced bone density, with severe porosis of the knees and tibia. Bone scintigraphy revealed a systemic demineralization. Since the age of 25, the patient suffered a gradual increase in the volume of the alveolar processes of the mandible and jaw and facial deformity. At age 40, she underwent surgery to reduce these lesions. Ultrastructural analysis of material from this intervention showed the presence of abundant fibroblasts in a fibrous stromal tissue with calcification foci surrounded by a thick fibrous layer and a subtle fibroblast layer. Hematological analysis was normal except for a high level of creatine phosphokinases (809 IU/l) and a slight elevation of alpha 2-globulin percentage (12.50%).

Subject V1 suffered spontaneous fractures starting at age 8 and involving several bones including the vertebrae, fibula, thighbones, radius/ulna, III right metatarsus, right iliac wing and hallux (Figure 2b). Moreover, after extraction of an impacted wisdom teeth, the patient experienced some local complications and healing problems. Radiological analysis of the mandible and jaw evidenced a cotton-wool-like pattern of the bony structure of the mandible (Figure 2a). He underwent surgical revision of the extraction site and bone biopsy. Histological analysis of the biopsic sample showed analogous findings to patient V5's analysis (Figure 2c).

Accurate clinical information for the other four affected members is provided in Table 1.

Molecular analysis

Genetic analysis of the ANO5 gene in seven affected individuals revealed a heterozygous mutation in exon 15 (c.1538C>T), which causes the substitution of threonine in position 513 with isoleucine (p.Thr513Ile) (Figure 3a). This variation was found to perfectly segregate with the disease, and was not present in dbSNP or 1000genomes databases, or in 400 chromosomes from healthy subjects. Moreover, it is defined as 'possibly damaging' by PolyPhen-2, with a score of 0.798. The residue is conserved in many but not all mammalian species, with some orthologs having an alanine in this position instead of threonine (Figure 3b). Interestingly, a threonine in the same position is also present in human ANO1 and ANO2.

Functional studies

As other members of the anoctamin family are plasma membrane Ca²⁺-activated Cl⁻ channels, we asked ourselves whether ANO5 expression is also associated with a similar activity. The ANO5 coding sequence, cloned from Saos-2 cells, was transiently expressed in HEK-293 cells together with the halide-sensitive YFP. In parallel, we transfected ANO1 (TMEM16A) alone or in combination with ANO5. Cells transfected with ANO1, and acutely stimulated with ionomycin to increase intracellular free Ca²⁺ concentration, showed a fast drop in cell fluorescence caused by a large I⁻ influx through

Figure 2 (a) Orthopantomography of patient V1: overgrown bone of both jaws with areas of cotton-wool-like pattern in the alveolar regions. (b) Thickening of diaphyseal cortex of the femur (long tubular bone) with fracture. (c) Histologic findings of the mandible in patient V1: abundant cell population represented by fibroblasts immersed in a fibrous stroma consisting of normal collagen fibrils; focally calcified areas bordered by thin fibroblasts, arranged circumferentially to the calcified area and separated from it by a thick layer of fibrous tissue.

Figure 3 (a) Alignment of the amino-acid sequences of different mammalian species showing that threonine 513 is conserved in most but not all of these species. (b) Chromatogram alignment showing the heterozygous c.1538C>T in an affected individual (lower panel) and the wt sequence in an unaffected individual (upper panel).

Ca^{2+} -activated Cl^- channels (Figure 4). A similar activity was also observed in cells co-transfected with *ANO1* plus *ANO5*. The assay could also detect the activity of *ANO2* (TMEM16B), which has a nearly 10-fold lower Ca^{2+} -sensitivity relative to *ANO1*.^{7,9,10} As expected for a channel that requires high (micromolar) intracellular

Ca^{2+} to be activated, *ANO2*-transfected cells showed a lower rate of anion transport (Figure 4). In contrast, anion transport in cells transfected with *ANO5* alone was insensitive to stimulation with ionomycin and comparable to that of mock-transfected cells (Figure 4).

Figure 4 Evaluation of Ca^{2+} -dependent anion transport. Summary of the maximum quenching rate, reflecting halide transport, for cells transfected with indicated plasmids with and without ionomycin. The bars report mean $\pm$ SEM of three to six separate experiments. The Ca^{2+} -dependent anion transport for cells transfected with *ANO1*, *ANO2* and *ANO1* plus *ANO5* was significantly higher than that measured in mock-transfected cells ($P < 0.01$).

DISCUSSION

In this study, we analyzed the coding region and 5' untranslated region of the *ANO5* gene in a large family affected with a disorder showing features characteristic of GDD, and found a novel heterozygous c.1538C>T mutation causing the p.Thr513Ile amino-acid substitution. This mutation, never before described, segregates with the disease within the family, is defined as 'possibly damaging' by PolyPhen-2 and is not present in our group of 200 healthy subjects. Moreover, Tmpred, a tool for prediction of transmembrane domains, suggests that p.Thr513Ile may affect the structure of the protein causing the fourth trans-membrane domain to start from residue 513 instead of 514. Taken together, this data suggests that threonine 513 is an important residue and that p.Thr513Ile may affect the function of *ANO5*.

Until now, only two mutations have been described in GDD families and they both affect the same cysteine residue in position 356 (p.C356G and p.C356R).⁴ This residue is predicted to be in the first extracellular loop, whereas threonine 513 is the second residue of the predicted fourth trans-membrane domain.

It is important to note that *ANO5* mutations have also been found in two recessive diseases that are very different from GDD: LGMD2L and distal MMD3 muscular dystrophies.^{21–24} Nineteen different mutations have been identified in homozygosity or compound heterozygosity. Such mutations include six variants causing a frameshift and premature truncation of the protein, 10 missense mutations, 1 splice site mutation causing an in-frame deletion of 24 residues and 2 mutations in hypothetical splice sites whose effects have not been verified at the cDNA level (Table 2). Some of these mutations have also been identified in subjects with asymptomatic hyperCKemia, exercise intolerance and myalgia, calf hypertrophy and

amyloidosis,^{22,23,25} suggesting a wide range of *ANO5*-related phenotypes. Mutations are spread throughout the *ANO5* gene, with no apparent trend in their position with regard to specific motifs, transmembrane domains or cytoplasmic versus extracellular regions of the protein.¹⁹ Truncating mutations trigger nonsense-mediated mRNA decay, suggesting loss-of-function due to deficient protein expression.¹⁷ Loss-of-function is also presumed for missense mutations identified in LGMD2L and MMD3.

Dominant missense mutations in GDD may reflect a gain-of-function effect predominantly in skeletal tissues.^{4,17} Alternatively, GDD mutations may exert a dominant negative effect, for instance on *ANO5* oligomerization. In 2011, Sheridan *et al* demonstrated that *ANO1* exists as a homodimer,²⁶ and we cannot exclude the possibility that other anoctamins, including *ANO5*, may have a similar oligomeric nature that may be disrupted by mutations in critical residues. Whereas cysteine 356 is absolutely conserved along evolution and can therefore be expected to be functionally important, threonine 513 has a lower degree of conservation. However, these mutations underlie very similar phenotypes, with no evident clinical heterogeneity. In fact, looking at the patient described by Tsutsumi *et al* in 2004,⁴ we observe the same jaw lesions causing facial deformities with propensity for jaw infection, presenting as purulent discharge from the gum, mobility of teeth and insufficient healing. At an extragnathic skeletal level we observed overlapping manifestations, such as bone fragility and thickening of tubular bones. Moreover, identical histological findings of the jaw lesions are described in our and Tsutsumi's patients and no abnormalities in non-skeletal tissues were reported in either set of patients. The very similar clinical manifestations induced by C356 and T513 mutations indicates a critical role for the two residues in *ANO5* function in bone cells. T513 is predicted to reside in the fourth transmembrane domain of anoctamins. Its substitution with a bulky hydrophobic residue may change the conformation of this region and its interaction with other *ANO5* domains. Analysis with 'Polyphen-2' defines this mutation as 'possibly damaging' and we believe that *in silico* modeling studies of protein structure may be useful to predict whether residue 513 could be important for protein folding or interaction with other proteins, and shed light on possible pathogenic mechanisms for the mutation identified in this study.

The function of *ANO5* is presently unknown. The similar membrane topology and the conservation of the putative pore region within the anoctamin family is compatible with a role for all anoctamins as channels, or components thereof, albeit with distinct properties and distribution. Our functional data demonstrate that transiently expressed *ANO5*, unlike *ANO1* and *ANO2*, does not support ionomycin-stimulated $\text{I}^{-}/\text{Cl}^{-}$ exchange in intact cells, adding weight to the hypothesis that *ANO5* may not function as a plasma membrane Ca^{2+} -activated chloride channel.^{11,12} However, our data do not preclude activation of membrane currents undetectable with the YFP-based assay, activation by other stimuli independent of Ca^{2+} or a possible localization and function of *ANO5* on intracellular membranes. Indeed, endogenous *ANO5* in musculoskeletal tissues is reported to be predominantly present in intracellular membrane fractions, and to a lesser degree on the plasma membrane.¹⁷ Following heterologous expression, *ANO5* is found on the endoplasmic reticulum.^{4,25} However, the correct localization and function of the endogenous protein may require additional factors present in bone or muscle cells. Specific antibodies against *ANO5* are needed to establish its subcellular localization, and transport studies on musculoskeletal cells or on specific subcellular compartments may help elucidate its potential role as a channel in these cells.

Table 2 ANO5 mutations discovered in patients showing different phenotypes

Mutation	Domain	Phenotype	Reference
c.1538C>T	p.Thr513Ile	Fourth transmembrane domain (TMD)	Bone disease
c.1067T>G	p.Cys356Gly	Extracellular, between first and second TMD	Bone disease
c.1067T>A	p.Cys356Arg	Extracellular, between first and second TMD	Bone disease
c.172C>T	p.Arg58Trp	Cytoplasmic tail N-terminal	Muscular disease
c.191dupA	p.Asn64LysfsX15	Cytoplasmic tail N-terminal	Muscular disease
c.242A>G	p.Asp81Gly	Cytoplasmic tail N-terminal	Muscular disease
c.295-1G>A	^a	Cytoplasmic tail N-terminal	Muscular disease
c.692G>T	p.Gly231Val	Cytoplasmic tail N-terminal	Muscular disease
c.1295C>G	p.Ala432GlyfsX49	Cytoplasmic, between second and third TMD	Muscular disease
c.1391delC_insAT	p.Ala464AspfsX30	Third TMD	Muscular disease
c.1407+5G>A	p.Glu445-Trp469del	Cytoplasmic and third TMD	Muscular disease
c.1502delT	p.Phe507SerfsX6	Extracellular, between third and fourth TMD	Muscular disease
c.1627dupA	p.Met543AsnfsX11	Cytoplasmic, between fourth and fifth TMD	Muscular disease
c.1643 C>T	p.Thr548Ile	Cytoplasmic, between fourth and fifth TMD	Muscular disease
c.1733T>C	p.Phe578Ser	Fifth TMD	Muscular disease
c.1899-4A>G	^a	Extracellular, between fifth and sixth TMD	Muscular disease
c.1965G>C	p.Trp655Cys	Extracellular, between fifth and sixth TMD	Muscular disease
c.2018A>G	p.Tyr673Cys	Extracellular, between fifth and sixth TMD	Muscular disease
c.2101A>G	p.Asn701Asp	Cytoplasmic, between sixth and seventh TMD	Muscular disease
c.2141C>G	p.Thr714Ser	Cytoplasmic, between sixth and seventh TMD	Muscular disease
c.2272C>T	p.Arg758Cys	Extracellular, between seventh and eighth TMD	Muscular disease
c.2311_2312delCA	p.Gln771AlafsX8	Extracellular, between seventh and eighth TMD	Muscular disease

^aMutation effect not verified at cDNA level.

The involvement of ANO5 in GDD, as well as in LGMD2L and MMD3 muscular dystrophies, suggests an important role in the development and differentiation of both skeletal muscle and bone. During murine embryogenesis, ANO5 is expressed in myotomal and sclerotomal somites with a differential spatiotemporal pattern, and in adult mice is abundant in cardiac and skeletal muscle, and in growth-plate chondrocytes and osteoblasts in bone.¹⁷ ANO5 expression is upregulated during myogenic differentiation in murine pluripotent mesenchymal precursor cells and is downregulated during osteoblastic differentiation, suggesting distinct regulatory mechanisms may govern ANO5 expression in muscular and skeletal tissues.¹⁶ Alternative splicing results in multiple ANO5 isoforms with potentially distinct transmembrane topology and subcellular localization, and this may contribute to the diverse roles of the protein in different tissues.¹⁶ It is worth noting that northern blot analysis revealed a smaller 3.5 kb transcript in bone compared with the 7.8-kb predicted transcript seen in skeletal muscle.¹⁶

Interestingly, serum CK levels in patient V5 were higher than normal and no other apparent muscle phenotype was observed. In contrast, CK levels in other affected individuals were in the normal range. Elevated CK also occurs in subjects with ANO5 recessive mutations including LGMD2L and MMD3 patients, as well as in asymptomatic carriers.^{23–25} Interestingly, MMD3 patient fibroblasts have defective membrane repair following cell wounding, suggesting that ANO5 may have a physiological role in this process.²⁷ Defective repair of the sarcolemma following contraction-induced mechanical stress may account for elevated serum CK levels in ANO5-related muscular dystrophy patients. The scarcity of patients with the GDD phenotype, however, means that we cannot draw firm conclusions as to whether the mutation causing GDD may also determine the increased level of serum CK in patient V5.

Disease-causing mutations have been described in two other members of the anoctamin family in addition to ANO5. Recently,

Vermeer *et al*²⁸ found mutations in *ANO10*, causing an autosomal recessive cerebellar ataxia. Four different mutations were found in three affected families: p.Leu384fsX90 and p.Leu535X in the hypothetical second and third cytoplasmic loops, respectively; c.1476+1G>T affecting the second extracellular loop and p.Leu510Arg in the fifth trans-membrane domain.²⁸ Moreover Suzuki *et al*¹⁵ and Castoldi *et al*²⁹ described mutations in *ANO6* as causative for Scott Syndrome, an inherited bleeding disorder. Three different mutations have been observed in affected individuals: one involving the acceptor splice site in intron 12, which causes the skipping of exon 13 and premature termination of the protein at the third trans-membrane segment;¹⁵ one involving the donor splice site at intron 6, resulting in an internal deletion of 212–249 residues within the N-terminal cytoplasmic tail, and one single base insertion in exon 11, which is predicted to cause the truncation of the protein between the second and third trans-membrane domain.²⁹

Apart from ANO5, only these members of the anoctamin family are known to be involved in diseases. The genetic heterogeneity and the still-unknown function of ANO5 make it difficult to unravel the molecular pathophysiology of GDD. Although functional studies are necessary, the findings of this third mutation may help in this unraveling process and in the understanding of the anoctamins' function and role in these different diseases, characterized by a very variable phenotype.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We acknowledge the Italian Ministry of Health, Strategic Program, RFPS-4-631972 'Genetic Bases of Birth Defects' for the financial support. We remember Dr Gianni Camera who passed away 7 years ago. He had in charge the family, characterized at clinical level some of the affected members and

collected all the samples. We thank him for encouraging this study and for his enthusiastic attitude he always shared with us.

URL

PolyPhen-2: <http://genetics.bwh.harvard.edu/pph2/>

TmPred: http://www.ch.embnet.org/software/TMPRED_form.html

- 1 Ahluwalia J, Ly JQ, Norman E, Costello Jr RF, Beall DP: Gnathodiaphyseal dysplasia. *Clin Imaging* 2007; **31**: 67–69.
- 2 Akasaka Y, Nakajima T, Koyama K, Furuya K, Mitsuka Y: Familial cases of new systemic bone disease, hereditary gnathodiaphyseal sclerosis. *Nippon Seikeigeka Gakkai Zasshi* 1969; **43**: 381–394.
- 3 Riminucci M, Collins MT, Corsi A *et al*: Gnathodiaphyseal dysplasia: a syndrome of fibro-osseous lesions of jawbones, bone fragility, and long bone bowing. *J Bone Miner Res* 2001; **16**: 1710–1718.
- 4 Tsutsumi S, Kamata N, Vokes TJ *et al*: The novel gene encoding a putative transmembrane protein is mutated in gnathodiaphyseal dysplasia (GDD). *Am J Hum Genet* 2004; **74**: 1255–1261.
- 5 Katoh M, Katoh M: GDD1 is identical to TMEM16E, a member of the TMEM16 family. *Am J Hum Genet* 2004; **75**: 927–928.
- 6 Yang YD, Cho H, Koo JY *et al*: TMEM16A confers receptor-activated calcium-dependent chloride conductance. *Nature* 2008; **455**: 1210–1215.
- 7 Schroeder BC, Cheng T, Jan YN, Jan LY: Expression cloning of TMEM16A as a calcium-activated chloride channel subunit. *Cell* 2008; **134**: 1019–1029.
- 8 Caputo A, Caci E, Ferrera L *et al*: 2008TMEM16A, a membrane protein associated with calcium-dependent chloride channel activity. *Science* 2008; **322**: 590–594.
- 9 Stohr H, Heisig JB, Benz PM *et al*: TMEM16B, a novel protein with calcium-dependent chloride channel activity, associates with a presynaptic protein complex in photoreceptor terminals. *J Neurosci* 2009; **29**: 6809–6818.
- 10 Stephan AB, Shum EY, Hirsh S, Cygnar KD, Reisert J, Zhao H: ANO2 is the ciliary calcium-activated chloride channel that may mediate olfactory amplification. *Proc Natl Acad Sci USA* 2009; **106**: 11776–11781.
- 11 Schreiber R, Uliyakina I, Kongsuphol P *et al*: Expression and function of epithelial anoctamins. *J Biol Chem* 2010; **285**: 7838–7845.
- 12 Duran C, Qu Z, Osunkoya AO, Cui Y, Hartzell HC: ANOs 3–7 in the Anoctamin/TMEM16 Cl⁻ channel family are intracellular proteins. *Am J Physiol* 2012; **302**: C482–C493.
- 13 Milenkovic VM, Brockmann M, Stöhr H, Weber BH, Strauss O: Evolution and functional divergence of the anoctamin family of membrane proteins. *BMC Evol Biol* 2010; **10**: 319.
- 14 Martins JR, Faria D, Kongsuphol P, Reisch B, Schreiber R, Kunzelmann K: Anoctamin 6 is an essential component of the outwardly rectifying chloride channel. *Proc Natl Acad Sci USA* 2011; **108**: 18168–18172.
- 15 Suzuki J, Umeda M, Sims PJ, Nagata S: Calcium-dependent phospholipid scrambling by TMEM16F. *Nature* 2010; **468**: 834–838.
- 16 Tsutsumi S, Inoue H, Sakamoto Y, Mizuta K, Kamata N, Itakura M: Molecular cloning and characterization of the murine gnathodiaphyseal dysplasia gene GDD1. *Biochem Biophys Res Commun* 2005; **331**: 1099–1106.
- 17 Mizuta K, Tsutsumi S, Inoue H *et al*: Molecular characterization of GDD1/TMEM16E, the gene product responsible for autosomal dominant gnathodiaphyseal dysplasia. *Biochem Biophys Res Commun* 2007; **357**: 126–132.
- 18 Bolduc V, Marlow G, Boycott KM *et al*: Recessive mutations in the putative calcium-activated chloride channel anoctamin 5 cause proximal LGMD2L and distal MMD3 muscular dystrophies. *Am J Hum Genet* 2010; **86**: 213–221.
- 19 Mahjneh I, Jaiswal J, Lamminen A *et al*: A new distal myopathy with mutation in anoctamin 5. *Neuromuscul Disord* 2010; **20**: 791–795.
- 20 Ferrera L, Caputo A, Ubbly I *et al*: Regulation of TMEM16A chloride channel properties by alternative splicing. *J Biol Chem* 2009; **284**: 33360–33368.
- 21 Hicks D, Sarkozy A, Muelas N *et al*: A founder mutation in anoctamin 5 is a major cause of limb-girdle muscular dystrophy. *Brain* 2011; **134**: Pt 1 171–182.
- 22 Deschauer M, Joshi PR, Gläser D, Hanisch F, Stoltenburg G, Zierz S: Muscular dystrophy due to mutations in anoctamin 5: Clinical and molecular genetic findings. *Nervenarzt* 2011; **82**: 1596–1603.
- 23 Schessl J, Kress W, Schoser B: Novel ANO5 mutations causing hyper-CK-emia, limb girdle muscular weakness and miyoshi type of muscular dystrophy. *Muscle Nerve* 2012; **45**: 740–742.
- 24 Penttilä S, Palmio J, Suominen T *et al*: Eight new mutations and the expanding phenotype variability in muscular dystrophy caused by ANO5. *Neurology* 2012; **78**: 897–903.
- 25 Milone M, Liewluck T, Winder TL, Pianosi PT: Amyloidosis and exercise intolerance in ANO5 muscular dystrophy. *Neuromuscul Disord* 2011; **22**: 13–15.
- 26 Sheridan JT, Worthington EN, Yu K, Gabriel SE, Hartzell HC, Tarran R: Characterization of the oligomeric structure of the Ca²⁺-activated Cl⁻ channel Ano1/TMEM16A. *J Biol Biochem* 2011; **286**: 1381–1388.
- 27 Jaiswal JK, Marlow G, Summerill G *et al*: Patients with a non-dysferlin Miyoshi myopathy have a novel membrane repair defect. *Traffic* 2007; **8**: 77–88.
- 28 Vermeer S, Hoischen A, Meijer RPP *et al*: Targeted next-generation sequencing of a 12.5 Mb homozygous region reveals ANO10 mutations in patients with autosomal-recessive cerebellar ataxia. *Am J Hum Genet* 2010; **87**: 813–819.
- 29 Castoldi E, Collins PW, Williamson PL, Bevers EM: Compound heterozygosity for 2 novel TMEM16F mutations in a patient with Scott syndrome. *Blood* 2011; **117**: 4399–4400.