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# Novel and recurrent *TRPV4* mutations and their association with distinct phenotypes within the *TRPV4* dysplasia family

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## ABSTRACT

**Background** Mutations in *TRPV4*, a gene that encodes a Ca<sup>2+</sup> permeable non-selective cation channel, have recently been found in a spectrum of skeletal dysplasias that includes brachyolmia, spondylometaphyseal dysplasia, Kozlowski type (SMDK) and metatropic dysplasia (MD). Only a total of seven missense mutations were detected, however. The full spectrum of *TRPV4* mutations and their phenotypes remained unclear.

**Objectives and methods** To examine *TRPV4* mutation spectrum and phenotype–genotype association, we searched for *TRPV4* mutations by PCR-direct sequencing from genomic DNA in 22 MD and 20 SMDK probands.

**Results** *TRPV4* mutations were found in all but one MD subject. In total, 19 different heterozygous mutations were identified in 41 subjects; two were recurrent and 17 were novel. In MD, a recurrent P799L mutation was identified in nine subjects, as well as 10 novel mutations including F471del, the first deletion mutation of *TRPV4*. In SMDK, a recurrent R594H mutation was identified in 12 subjects and seven novel mutations. An association between the position of mutations and the disease phenotype was also observed. Thus, P799 in exon 15 is a hot codon for MD mutations, as four different amino acid substitutions have been observed at this codon; while R594 in exon 11 is a hotspot for SMDK mutations.

**Conclusion** The *TRPV4* mutation spectrum in MD and SMDK, which showed genotype–phenotype correlation and potential functional significance of mutations that are non-randomly distributed over the gene, was presented in this study. The results would help diagnostic laboratories establish efficient screening strategies for genetic diagnosis of the *TRPV4* dysplasia family diseases.

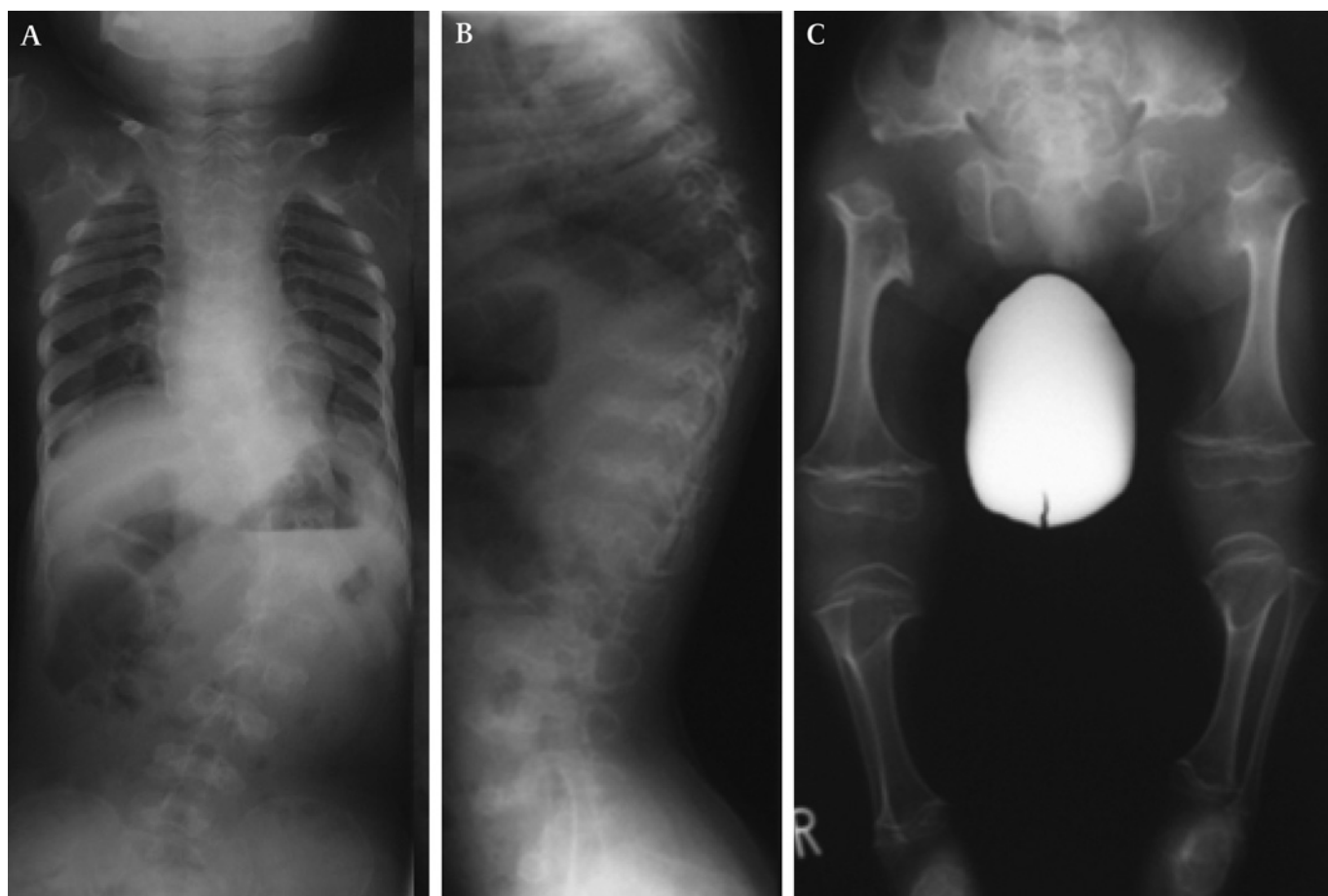
Metatropic dysplasia (MD; OMIM 156530) is a severe skeletal dysplasia. “Metatropic” is derived from the Greek and refers to the age-dependent evolution of body proportion in MD, changing from short limb at birth to short trunk in childhood as a result of progressive kyphoscoliosis.<sup>1</sup> Affected individuals present with narrow thorax, prominent joints and occasionally tail-like coccygeal appendage (caudal tail). The radiological hallmarks of MD include narrow thoracic cage with short ribs, severe platyspondyly with elongated vertebral bodies, flared ilia with horizontal acetabula and occasionally

supra-acetabular notches and marked metaphyseal enlargement of the long bones, leading to a dumbbell appearance (figure 1).<sup>2</sup> MD has so far been considered to be genetically heterogeneous. While the majority was felt to be autosomal dominant, a subset of patients with severe phenotypes was presumed to be inherited as autosomal recessive.<sup>3</sup>

Spondylometaphyseal dysplasia, Kozlowski type (SMDK; OMIM 1842522), is an autosomal dominant skeletal dysplasia characterised by short trunk with platyspondyly and metaphyseal dysplasia (metaphyseal irregularity and flaring) of the long bones (figure 2).<sup>4 5</sup> Progressive kyphoscoliosis is common, and the ilium is broad and occasionally flared. A diagnostic skeletal alteration of SMDK is overfaced pedicles that refer to broadened vertebral bodies extending beyond pedicles on antero-posterior radiograph of the spine (figure 2A).

Brachyolmia (BO) is a heterogeneous group of skeletal dysplasias characterised by short trunk and general platyspondyly without significant epiphysial, metaphyseal and diaphyseal changes in the long bones. Based on the mode of inheritance and radiographic features, three types have been described.<sup>6</sup> The autosomal dominant form of BO (OMIM 113500) shows platyspondyly that is particularly severe in the cervical spine, overfaced pedicles and broad ilia. These features resemble those of SMDK.

Autosomal dominant MD, SMDK and autosomal dominant BO are caused by heterozygous mutations in the gene encoding TRPV4 (transient receptor potential cation channel, subfamily V, member 4). TRPV4 is a calcium permeable nonselective cation channel.<sup>7</sup> Human TRPV4 is a protein of 871 amino-acids composed by a proline-rich region, six ankyrin (ANK) repeats, six transmembrane (TM) domains and a calmodulin-binding domain (figure 3). A putative cation-permeable pore is located between the fifth and sixth TM domains.<sup>8</sup> TRPV4 is activated by a variety of physical and chemical stimuli, including heat, mechano-stimuli, endogenous substances such as arachidonic acid and synthetic alpha-phorbol derivatives. TRPV4 is involved in many different cellular functions; it has an important role in differentiation of chondrocytes and terminal differentiation of osteoclasts via calcium influx.<sup>9 10</sup>



**Figure 1** Radiographs of metatropic dysplasia (M8). (A) AP (antero-posterior) view of trunk, (B) lateral view of the thoracolumbar spine and (C) AP view of the lower extremities at age 3 years. There are thoracolumbar kyphoscoliosis, severe platyspondyly with overfaced pedicles and flared ilia with supra-acetabular notches. Significantly flared metaphyses of the long bones present a dumbbell deformity. Epiphyseal ossification of the proximal femora is significantly retarded.

Through linkage analysis followed by a candidate gene approach, Rock *et al* identified two *TRPV4* mutations in autosomal dominant BO.<sup>11</sup> Because of radiological similarities between BO and SMDK, Krakow *et al* subsequently tested *TRPV4* in patients with SMDK and found three different heterozygous mutations. Then, because of radiographic similarities between MD and SMDK, they further examined *TRPV4* and found two mutations in autosomal dominant MD.<sup>12</sup> Thus, *TRPV4* is the causative gene of a spectrum of disorders that constitute a bone dysplasia family including autosomal dominant BO, SMDK and autosomal dominant MD. However, because only seven distinct mutations have been reported so far (three in SMDK and two in BO and MD, respectively)<sup>11 12</sup> (figure 3), the spectrum of *TRPV4* mutation remains unclear, as well as the range of phenotypes caused by *TRPV4* mutations.

To further evaluate these questions, we searched for *TRPV4* mutations in a total of 42 families with MD and SMDK. We detected 19 kinds of heterozygous mutations in 41 patients and found two mutational hotspots in *TRPV4*. We also found an association between location of mutations and disease phenotype.

## SUBJECTS AND METHODS

### Patients

MD and SMDK patients were enrolled from the participating institutions. The study was approved by the ethical committee of RIKEN and participating institutions and informed consent

was obtained from all subjects. Clinical assessments for the patients were performed by their clinicians (Supplementary Table 1). The radiological features were reviewed by two pediatric radiologists (O.K. and G.N.) and by a paediatrician and a geneticist (A.S.-F. and S.U.) separately, and then discussed to consensus conclusion (supplementary Table 2).

The radiographic criteria for MD included wafer-like thin or diamond-shaped vertebral bodies in infancy and marked platyspondyly with progressive spinal deformities (scoliosis and/or kyphosis) in childhood (figure 1). The criteria also included flared iliac wings, flat acetabula and supra-acetabular notches in combination and dumbbell- or halberd-shaped metaphyseal widening of the long bones, particularly in the femora (figure 1). Other manifestations, including metaphyseal irregularities, epiphyseal dysplasia, brachydactyly and delayed carpal bone age, were considered; however, these findings only partially contributed to the diagnosis because they were age-dependent and difficult to evaluate in younger patients.

The criteria for SMDK included a variable degree of platyspondyly (according to the age), overfaced pedicles, flat acetabula with/without flared iliac wings and supra-acetabular notches, flared metaphyses of the long bones without dumbbell shape, metaphyseal irregularities with almost normal epiphyses and almost normal short tubular bones (figure 2). Metaphyseal irregularities were assessable only in pre-pubertal patients. Carpal age was evaluated because delayed bone age has been emphasised as an essential finding of SMDK. BO was defined as the condition

## Short report

**Figure 2** Radiographs of spondylometaphyseal dysplasia, Kozlowski type (SMDK) (S6). (A) AP and (B) lateral views of trunk at age 5 years and 9 months. Thoracolumbar kyphoscoliosis, platyspondyly with overfaced pedicles, flared ilia with supraacetabular notches are identical with those of metatropic dysplasia. However, mild flaring of the long bones contrasts with dumbbell deformity seen in metatropic dysplasia. Note metaphyseal dysplasia of the proximal femora. This patient had the common SMDK mutation, R594H.



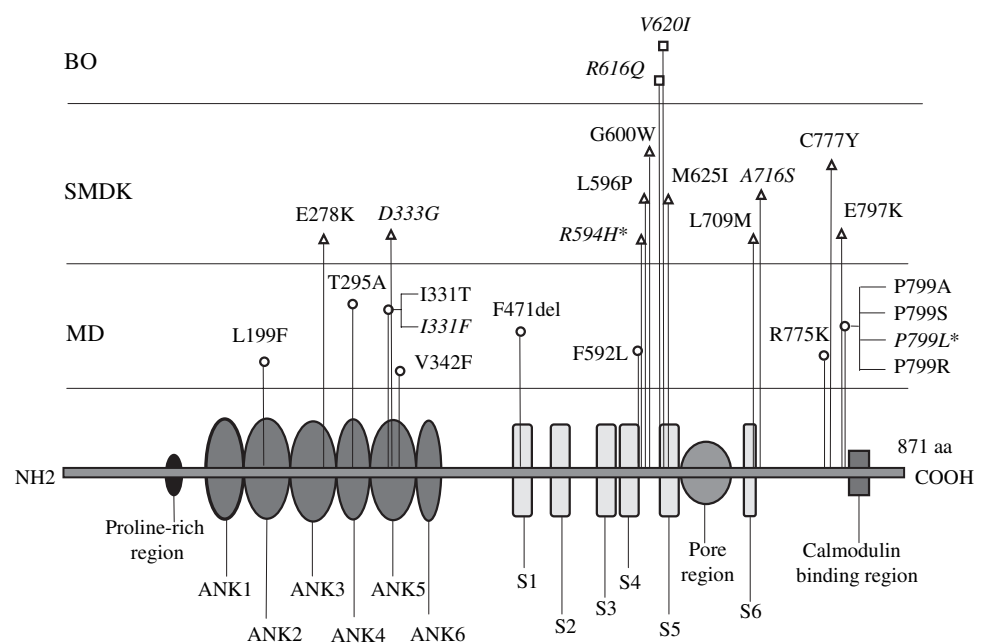
that showed platyspondyly with overfaced pedicles and broad ilia, but no overt metaphyseal changes (flaring and irregularity).

### Mutation search

Genomic DNA was extracted by standard procedures from peripheral blood or by Isohair kit (NIPPON GENE, Wako, Japan)

from nail and hair. Exon sequence of *TRPV4* with its flanking intron sequence was amplified by PCR from genomic DNA. PCR products were sequenced directly by using an ABI Prism 3700 automated sequencer (PE Biosystems, Foster City, CA, USA). PCR primer sequence is available on request, and the same primer was used for sequencing. For confirmation of novel

**Figure 3** A diagram of human *TRPV4* protein and location of mutations. ANK: ankyrin repeat region. S1–S6: transmembrane regions 1–6. BO: brachyolmia. SMDK: spondylometaphyseal dysplasia, Kozlowski type. MD: metatropic dysplasia. \*Common recurrent mutations. Mutations found in the previous studies are in italic.





mutations in sporadic cases, genomic DNA from the unaffected parents was sequenced for the corresponding regions when parents' samples were available. The molecular analysis was performed independently in the two laboratories in Tokyo and in Freiburg.

### Restriction fragment length polymorphism

PCR-restriction fragment length polymorphism (RFLP) method was used to confirm mutations in two hotspot codons, R594 and P799. c.1781G→A creates the *NcoI* (TAKARA BIO, Dalian, China) restriction site, and c.2395C→T/G and c.2396C→T/G abolishes *SmaI* (TAKARA BIO) restriction site, respectively. PCRs were the same as those for sequencing. We prepared the reaction mixture according to the manufactures of the two enzymes and incubated it at 37°C overnight. Digested PCR products were electrophoresed in 4% agarose gel (3% NuSieve GTG Agarose (Lonza, Rockland, Maine, USA) and 1% SeaKem LE Agarose (BMA, Rockland, Maine, USA)).

### RESULTS

A total of 42 probands were included in the study. There were 14 Koreans, 14 Europeans, 10 Japanese, two Turks and one Indian. All MD cases were sporadic and four SMDK cases were familial. All were from non-consanguineous marriages. The clinical and radiological findings of the patients are summarised in supplementary tables 1 and 2. The phenotypes comprised 22 MD and 20 SMDK. In general, a radiological diagnosis of bone dysplasias rests on the overall pattern recognition of skeletal changes rather than a single radiological sign; after all, however, presence/absence of dumbbell- or halberd-shaped femora ascertained distinction between MD and SMDK (supplementary table 2). Other radiological signs were shared by both disorders. All subjects had flat acetabular roofs. Two SMDK patients (S12 and S18) did not show overt metaphyseal changes (Supplementary figure 1) and were considered to be of intermediate severity between SMDK and BO. Kozłowski *et al* (1982) reported SMDK with subtle metaphyseal irregularity.<sup>4</sup> All other subjects had metaphyseal changes (flaring and/or irregularity) (supplementary table 2).

Detailed radiographic review revealed several unexpected findings. Narrow thorax, prominent joints and coccygeal tail are considered to be clinical hallmarks of MD; however, only prominent joints were consistently found in MD, and these features were also occasionally found in SMDK (supplementary table 1). Evolution of body proportion with age, another hallmark of MD, was not essential; several post-pubertal MD patients showed short limbs, not short trunks. Delayed carpal age, a diagnostic criterion for SMDK, was not observed in half of SMDK patients. MD patients after infancy showed overfaced pedicles that were indistinguishable from those in SMDK patients. A small percentage of SMDK patients showed mild brachydactyly or mild epiphysial dysplasia/premature degenerative joint disease, yet, these cases were classified as SMDK based on the overall pattern of skeletal changes.

Mutations were found in 41 subjects (supplementary table 3). All subjects were at the heterozygous state for the mutation. We found 19 different *TRPV4* mutations; 17 were novel. All but c.1411\_1413delTTC (p.F471del) were missense mutations, and all affected evolutionally conserved amino acids. We examined *TRPV4* sequences in the parents of probands with novel mutations except for S15, and confirmed that mutations in sporadic cases were all *de novo*. In S15, the mother's DNA was unavailable, but the mutation, c.1787T→C (p.L596P), was not found in the father, nor in 80 unrelated ethnicity-matched controls. L596

is highly conserved between diverse species (supplementary figure 2) and the observed amino-acid change is non-conservative. In two familial cases (S16 and S18) where their mutations were not proven to be *de novo* in this and previous studies, the substituted amino acids were also highly conserved among diverse species (supplementary figure 2). An *in silico* analysis by PANTHER (<http://www.pantherdb.org/>) indicated mutations as probably damaging protein function. There were no inconsistencies in segregation of the mutations in the family members as far as we examined.

Eleven different mutations were detected in 21 MD patients (supplementary table 3). One recurrent mutation, c.2396C→T (p.P799L), was detected in nine patients. It was confirmed as *de novo* in one family. Ten kinds of novel mutations were found. Eight of them were found only once; c.2324G→A (p.R775K) and c.2396C→G (p.P799R) were both found in two patients. Mutations altering codon 799 were detected in 13 patients. In M22, no mutation was found in the coding region of *TRPV4*, 5'- and 3'-UTRs, nor flanking intron sequences.

Eight different *TRPV4* mutations were found in SMDK (supplementary table 3). All 20 SMDK patients had heterozygous mutations in *TRPV4*; 14/20 mutations were in exon 11. A recurrent mutation, c.1781G→A (p.R594H) was found in 12 patients. The mutation was not found in normal parents in two families, indicating that they were *de novo*. The patients who showed intermediate phenotypes of SMDK and BO had the recurrent mutation, c.1781G→A and a novel mutation, c.2125C→A, respectively.

### DISCUSSION

We found that *TRPV4* mutations in the MD and SMDK-disease spectrum include two mutational hotspots. We have observed the recurrent mutation c.1781G→A, which had been found in four SMDK patients in the previous study<sup>12</sup> and in 12 SMDK subjects in our cohort (supplementary table 3). The mutation was identified in various ethnic backgrounds. c.1781G→A is at present the most prevalent *TRPV4* mutation; 16/51 known cases carry this mutation. We also found that c.2396C→T is a recurrent mutation in MD (supplementary table 3). This mutation, found in one MD patient in the previous study,<sup>11</sup> was found in nine MD subjects with various ethnicities in our study. Knowledge of the mutation hot spots will enable the construction of an efficient screening system for *TRPV4* mutations and facilitate the molecular diagnosis of these diseases. The two RFLPs established in this study are useful tools for the screening of *TRPV4* mutations as they can capture ~60% (30/51) of *TRPV4* mutations.<sup>11 12</sup>

MD and SMDK mutations are clustered in specific exons. Exon 11 is a hot exon for SMDK mutations. 15 mutations were identified in this exon in our patient population and taken together with results of previous studies,<sup>11 12</sup> ~70% (18/26) of SMDK mutations were found in this exon. Only one exon-11 mutation has been identified in a MD patient. In contrast, exon 15 is a hot exon for MD mutations; ~60% (14/23) of MD mutations were found in this exon. There is only one exon-15 mutation in SMDK. All but one exon-15 mutation occurred at the same P799 codon; interestingly, four different mutations, leading to four different amino acid substitutions, were found at this "hot codon". In practice, these observations mean that we can prioritise specific *TRPV4* exons for MD and SMDK when searching for mutations. The locations of the mutations relative to the domains of the molecule (ANK, TM, cytoplasmic, etc.) did not show any consistent relation with phenotypes (figure 3).<sup>13</sup>

## Short report

Despite genetic homogeneity of SMDK, its phenotypic range was broad, particularly in the severity of metaphyseal dysplasia and in appearances of ilia. The variable phenotypes, even due to the same mutation, are in part attributable to age-related differences. Sequential radiological follow-up would address this issue. MD patients who had mutations in exon 15 showed milder radiographic changes in terms of rib shortening, platyspondyly and dumbbell deformity than those with mutations in other exons (figure 1 and supplementary figure 3). However, accurate delineation of the total phenotypic spectrum even among individuals with the common mutation would require further accumulation of cases with radiographs taken at standard ages.

Genetic heterogeneity of MD has long been in debate. Most investigators have believed that MD comprises lethal autosomal recessive and non-lethal autosomal dominant phenotypes.<sup>3</sup> On the other hand, other investigators have suggested that all cases represent a dominant form with phenotypic variability.<sup>2 14</sup> Our series of patients included individuals with a relatively mild SMDK/BO phenotype and individuals with quite severe, though non-lethal, MD; thus, the concept that all forms of MD may be the result of dominant mutations seems quite likely. We studied one MD patient (M22) who did not have a *TRPV4* mutation. The girl, unlike other affected children, has not shown epiphyseal dysplasia until now (supplementary figure 4). However, because she is still young, we have to follow-up her radiological evolution to determine whether her phenotype differs from those of other patients with *TRPV4* mutations (supplementary tables 1 and 2).

One MD patient had a 3-bp deletion in *TRPV4*. All other mutations in this study, as well as the seven mutations previously reported, are missense mutations.<sup>11 12</sup> The deletion mutation caused the loss of an evolutionally conserved amino-acid residue. From a previous functional study,<sup>11</sup> the molecular pathogenetic basis of *TRPV4*-pathies has been suggested to be a gain-of-function characterised by increased constitutive activity and elevated channel activation by a variety of mechanisms. It remains to be examined whether the deletion mutation would also cause a gain of *TRPV4* function.

In summary, we found *TRPV4* mutations in 41 of 42 patients with MD and SMDK, indicating genetic homogeneity for these disorders. We identified 17 different novel mutations, including the first deletion mutation of *TRPV4*. We confirmed that R594H is a common and recurrent mutation in SMDK and found that P799L is a common and recurrent mutation in MD. There were mutational hot spots for *TRPV4*: R594 in exon 11 is a hot spot exclusive for SMDK mutations, while P799 in exon 15 is a hot spot for MD mutations. Our study presented an initial picture for the *TRPV4* mutation spectrum in MD and SMDK. Further

studies are necessary to determine its complete picture and phenotypic extension of the *TRPV4* mutation.

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**Competing interests** None.

**Patient consent** Obtained.

**Ethics approval** This study was conducted with the approval of the Ethical Committee of RIKEN, Japan.

**Contributors** Jin Dai, Ok-Hwa Kim, and Tae-Joon Cho, these authors are contributed equally to the work.

## Key points

- ▶ *TRPV4* mutations were found in 40/41 subjects with MD and SMDK, including 17 kinds of novel mutations.
- ▶ Two mutation hot spots were identified for MD and SMDK, respectively.
- ▶ MD mutations were clustered at P799 in exon 15, while SMDK mutations were clustered at R594 in exon 11.
- ▶ Novel type of mutation other than missense mutation, 3-bp deletion was found in one MD patient.
- ▶ Our results would help diagnostic laboratories establish efficient screening strategies for genetic diagnosis of the *TRPV4* dysplasia family diseases.

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## Novel and recurrent *TRPV4* mutations and their association with distinct phenotypes within the *TRPV4* dysplasia family

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