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SHORT COMMUNICATION

Novel point mutations in *GDF5* associated with two distinct limb malformations in Chinese: brachydactyly type C and proximal symphalangism

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Abstract Growth/differentiation factor 5 (GDF5) is a secreted growth factor that plays a key regulatory role in embryonic skeletal and joint development. Mutations in the GDF5 gene can cause different types of skeletal dysplasia, including brachydactyly type C (BDC) and proximal symphalangism (SYM1). We report two novel mutations in the *GDF5* gene in Chinese families with distinct limb malformations. In one family affected with BDC, we identified a novel nonsense mutation, c.1461T > G (p.Y487X), which is predicted to truncate the GDF5 precursor protein by deleting 15 amino acids at its C-terminus. In one family with SYM1, we found a novel missense mutation, c.1118T > G (p.L373R), which changes a highly conserved amino acid in the prodomain of GDF5. We transfected COS-7 cells with retroviral constructs to express human wild-type or mutant GDF5 cDNAs. The mature GDF5 protein was detected, as in the wild-type, in supernatant derived from the p.L373R mutant GDF5 transfected cells, but not in the supernatant from the p.Y487X mutant transfected cells, indicating that the two mutations led to different fates of the mutant GDF5 proteins, thereby producing distinct limb phenotypes.

Keywords Growth/differentiation factor 5 · Brachydactyly type C · Proximal symphalangism · Nonsense mutation · Missense mutation

Introduction

Growth/differentiation factor 5 (GDF5), also known as cartilage-derived morphogenetic protein-1 (CDMP1), is a member of the bone morphogenetic protein (BMP) family and the transforming growth factor-beta (TGF-β) superfamily (Chang et al. 1994; Luyten 1997; Storm and Kingsley 1999). GDF5 precursor polypeptide contains 501 amino acids with a RRKRR polybasic proteolytic processing site at amino acids 377–381 and seven highly conserved cysteines at its C-terminus. It forms homodimers or heterodimers with other BMP partners linked by a single inter-chain disulfide bond. These dimers undergo proteolysis, producing an active mature GDF5 dimer that is secreted from the cell (Luyten 1997). It has been shown that GDF5 plays a key regulatory role in embryonic skeletal and joint development (Chang et al. 1994; Luyten 1997; Storm and Kingsley 1999). Mutations in the *GDF5* gene can cause several different types of skeletal dysplasia, including brachydactyly type A2 (MIM 112600), brachydactyly type C (BDC; MIM 113100), fibular hypoplasia and complex brachydactyly (Du Pan syndrome) (MIM 228900), Grebe type chondrodysplasia (MIM 200700), Hunter-Thompson type acromesomelic dysplasia (MIM 201250), multiple synostoses syndrome type 1 (MIM 186500) and proximal symphalangism (SYM1; MIM 185800) (Seemann et al. 2005; Polinkovsky

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et al. 1997; Faiyaz-Ul-Haque et al. 2002; Thomas et al. 1996, 1997; Dawson et al. 2006). Recently, +104T/C (rs143383), a single nucleotide polymorphism in the 5′-untranslated region of the *GDF5* gene, was found to show significant association with osteoarthritis (MIM 165720) in Asian and European populations (Miyamoto et al. 2007; Southam et al. 2007).

Brachydactyly refers to shortening of digits due to underdevelopment of the bones in the hands and/or feet. It usually occurs as an isolated autosomal dominant trait, but may also present as part of many genetic syndromes. On an anatomic and genetic basis, nonsyndromic brachydactyly has been classified into five main types, A-E. BDC is characterized by short middle phalanges of the second, third and fifth fingers, hypersegmentaion of the second and/or third finger, and shortening of the first metacarpal. In individuals with BDC, the fourth finger is generally the longest digit because it is rarely affected. Other phenotypes, such as hip dysplasia, Madelung deformity, polydactyly, short stature, and talipes valgus or equinivarus, have also been reported in individuals with BDC. By genetic linkage analysis, BDC was mapped to the chromosomal 20q11.2 region, which harbors the *GDF5* gene. Further genetic analyses in families with BDC demonstrated locus homogeneity and identified *GDF5* as the locus for BDC (Polinkovsky et al. 1997).

SYM1, also called Cushing symphalangism, refers to hereditary absence of the proximal interphalangeal joints caused by bony fusion of middle and proximal phalanges in the hands and feet. SYM1 is transmitted as an autosomal dominant trait and has been mapped to chromosomal region 17q21-q22 (Polymeropoulos et al. 1995). Gong and colleagues demonstrated that mutations in *NOG*, the gene encoding the BMP antagonist noggin protein, can lead to SYM1 (Gong et al. 1999). Recent identification of two gain-of-function mutations in the active mature domain of GDF5 established the link between SYM1 and the *GDF5* gene (Seemann et al. 2005; Wang et al. 2006).

In the present report, we describe two novel *GDF5* mutations, one nonsense mutation in the active mature domain in a large Chinese family with BDC and one missense mutation in the prodomain in a Chinese family with SYM1. We also showed different dimer formation and secretion of the mature GDF5 protein in COS-7 cells expressing the two mutant precursor polypeptides.

Methods

Mutation detection

Peripheral blood samples were collected from two Chinese families (Fig. 1), family 1 with BDC and family 2 with SYM1, after informed consent of participating family members and approval of Peking Union Medical College Institutional Review Board were obtained. Genomic DNA was extracted from blood samples using standard SDS-proteinase K-phenol/chloroform protocol. The two coding exons and their flanking intronic sequences of the *GDF5* gene were PCR-amplified and then subjected to automatic DNA sequencing after purification. The two primer pairs used were GDF5-1U1 (5-TTCTCTTTGGTGTCATT CAGCGGCT-3)/GDF5-1D1 (5-GAAAGCCCCTCCATTC ATGCAGAT-3) and GDF5-2U3 (5-ACACCCCTAATT GGTCGGCTAT-3)/GDF5-2D1 (5-GCTTATTGGAATCC CCTTTCACC-3) for exons 1 and 2, respectively. To confirm the mutation identified in family 1, an *Xba* I restriction site was introduced into the mutant allele using mismatch primer in a semi-nested PCR. Genomic DNA was first amplified using primers GDF5-2U1 (5-CCAC-GAATCATGCAGTCATCC-3) and GDF5-2D1. The resultant amplicon of 311 bp was used as template in the second round of PCR amplification using the mismatch primer: 5-TCTGCCAACAACGTGGTCTA-3 (the mismatch "C" is underlined) and GDF5-2D1 to create an *Xba* I restriction site (TCTA***G***A) (the "G" at nucleotide 1461 is

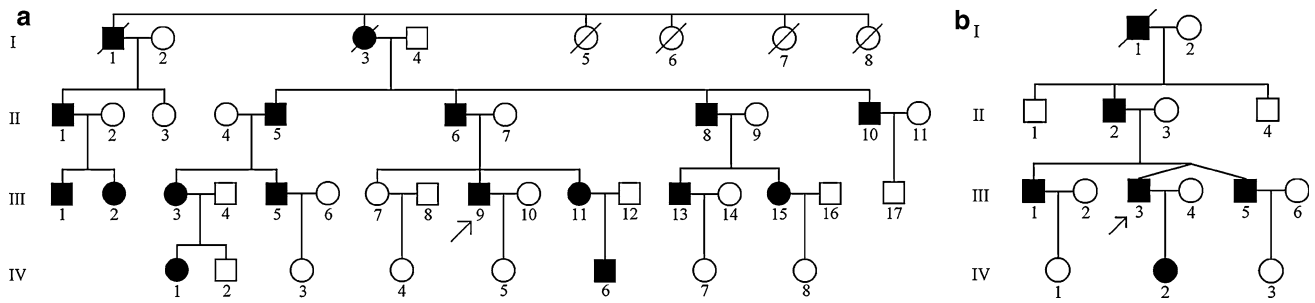

Fig. 1 Pedigrees of families with limb malformations. **a** Family 1 with brachydactyly type C (BDC). **b** Family with proximal symphalangism (SYM1). Filled symbols represent affected family members with BDC or SYM1, respectively, and open symbols represent family members with normal hands and feet. *Circles* and *squares* indicate females and males, respectively. The *arrows* identify the probands

in bold italic) in the mutant 1461G allele, but not in the normal 1461T allele (TCTATA). The amplicons of 199 bp derived from the semi-nested PCR were digested with restriction enzyme *Xba* I and separated by electrophoresis on 12% neutral polyacrylamide gel and displayed by routine silver staining. The mutation found in the SYM1 family was confirmed by *Msp* I restriction analysis of the PCR amplicons of 342 bp generated using primers GDF5-E2U (5′-TCCACGAGAAGGCCCTGTTC-3′) and GDF5-E2D (5′-GGATGACTGCATGATTCGTGG-3′). The *Msp* I digests were also separated and displayed as described above. Available family members and 50 unrelated normal controls were included in the above-mentioned restriction analyses. In family 2, an amplicon of 1,054 bp encompassing the entire coding region of the *NOG* gene was also produced by PCR using primers NOG-U1 (5-TCGC CCTGGAGTAATTTCGGAT-3) and NOG-D1 (5-GAA CTGGTTGGAGGCGGTGC-3), and sequenced.

Plasmid construction

The full-length GDF5 coding sequence was first obtained by PCR using the Marathon-Ready Human Fetal Brain cDNA (Clontech) as template. The primer pairs used in cDNA amplification were GDF5-RVU (5′-TATGTT AACGCCACCATGAGACTCCCCAAAC-3′) and GDF5-RVD (5′-AAGGATCCCTACCTGCAGCCACACGACT C-3′). The amplified cDNA fragment was cloned into the pLXSN retroviral vector at the *Hpa* I and *BamH* I sites and verified by sequencing. The 5′-GAACAAAAACTCATCT CAGAAGAGGATCTG-3′ sequence, encoding the widely used c-myc epitope EQKLISEEDL, was then inserted into the human *GDF5* cDNA between nucleotides 1,158 and 1,159 by PCR (Everman et al. 2002), to produce the pLmycGDF5^{WT}SN retroviral expression plasmid. The c.1461T > G and c.1118T > G mutations were introduced into the plasmid, resulting in the pLmycGDF5^{Y487X}SN and pLmycGDF5^{L373R}SN plasmids, respectively.

Cell culture, viral package, transduction and western blot

COS-7 and GP-293 cells were grown in DMEM with high glucose concentration supplemented with 10% fetal bovine serum. pLmycGDF5^{WT}SN, pLmycGDF5^{Y487X}SN and pLmycGDF5^{L373R}SN were cotransfected with pVSV into the packaging cell line GP-293 using Lipofectamine^{TM} 2000 Reagent (Invitrogen) to generate recombinant retroviruses containing LmycGDF5^{WT}SN, LmycGDF5^{Y487X}SN and LmycGDF5^{L373R}SN, respectively. Two days after transfection, viral supernatant was collected. The recombinant retroviruses were then used to infect COS-7 cells. Twelve hours after transduction, cells were subjected to G418 selection (1 mg/ml) for 2 weeks. The supernatant samples were collected and concentrated using Amicon Ultra-4 5 K centrifugal filter devices (Millipore). The concentrated samples were then separated by 15% SDS-polyacrylamide gel electrophoresis under non-reducing conditions and transferred to a PVDF membrane (Millipore). The membrane was stained using Ponceau red to confirm efficient transfer of proteins from the supernatant samples. After blocking in TBS (10 mM Tris, pH 7.5, 150 mM NaCl) containing 1% non-fat dried milk and 5% bovine serum albumin, blots were probed with the anti-c-Myc monoclonal antibody 9E10 (1:200) (Santa Cruz), followed by ImmunoPure Peroxidase Conjugated Goat Anti-Mouse IgG (Pierce), and then developed with the SuperSignal West Femto Maximum Sensitivity Substrate (Pierce).

Results

Clinical findings

We investigated two Han Chinese families with distinct limb malformations. Both families had affected females and male-to-male transmission (Fig. 1), consistent with autosomal dominant inheritance. Family 1 consists of 44 individuals in four generations, with 17 affected individuals (Fig. 1a). Three individuals, including the proband (III-9), III-11 and IV-6, were physically examined. They all exhibited bilateral shortening of the thumbs and fingers 2, 3 and 5. Hand radiograph in the proband revealed remarkable shortening of the middle phalanges of fingers 2, 3 and 5, and of the first metacarpals (Fig. 2a), a cardinal phenotypic feature of BDC. No obvious shortening of toes or metatarsals were observed. Noticeably, III-11 and IV-6 have severe palmoplantar keratoderma. Family 2 had six affected individuals in four generations (Fig. 1b). All five affected individuals who are still alive were available for phenotype evaluation. Radiographs were taken in four of them. On clinical examination, they all displayed generalized shortening of hands, absence of proximal interphalangeal creases of fingers 2–5, clinodactyly of the 5th fingers, and shortening of toes 3–5 (Fig. 2b, d). Three individuals (III-3, III-5 and IV-2 in Fig. 1b) showed short great toes and flat feet (Fig. 2d, f). Radiographs revealed a constant phenotypic feature in all four individuals: bony fusion between the proximal and middle phalanges of fingers 2–5 (complete in II-2, III-1 and III-3, and partial in IV-2), shortened first metacarpals and biphalangeal toes 2–5 (Fig. 2c,e). Short first metatarsals were apparent in III-3 and IV-2 (Fig. 2e). No individual in the families showed short stature or hearing loss.

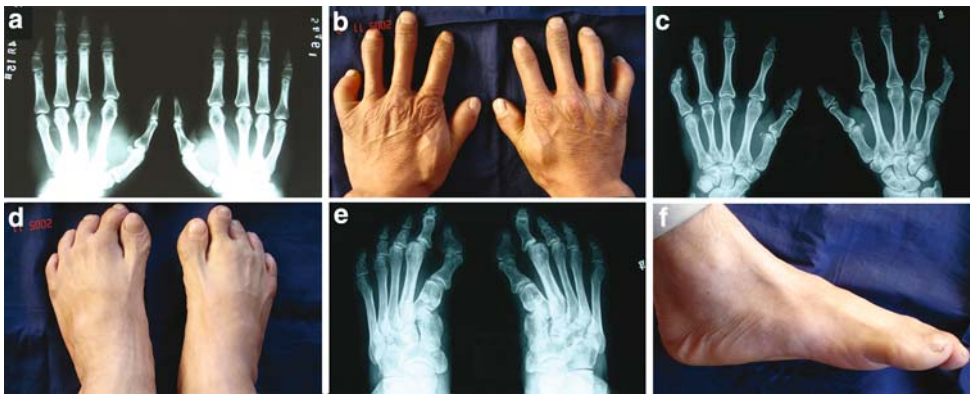

Fig. 2 Photographs and radiographs of the probands. **a** Hand radiograph of the proband in family 1 showing short second, third and fifth middle phalanges, and first metacarpals. **b**, **c** Hand photograph and radiograph of the proband in family 2. **d**, **e** Foot photograph and radiograph of the proband in family 2. **f** Photograph displaying flat foot in the proband in family 2

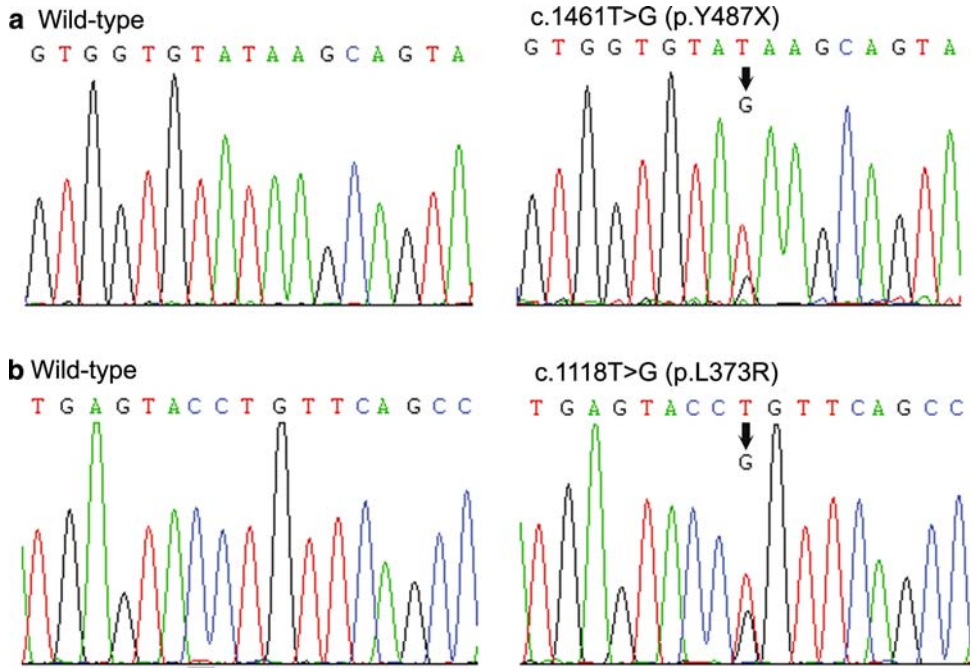

Fig. 3 Two novel GDF5 mutations in study families. **a** DNA sequence analysis demonstrating the presence of the nonsense mutation, c.1461T > G (p.Y487X), in the proband of family 1. **b** DNA sequence analysis demonstrating the presence of the missense mutation, c.1118T > G (p.L373R), in the proband of family 2

Identification of novel *GDF5* mutations

We searched for pathogenic mutations in the proband of family 1 (III-9 in Fig. 1a) by direct sequencing of the PCR-amplified DNA fragments spanning exons 1 and 2 of the *GDF5* gene. Sequencing of PCR amplicons generated by primers GDF5-2U3 and GDF5-2D1 revealed a novel nonsense mutation, c.1461T > G (p.Y487X), producing a premature stop codon at amino acid residue 487 (Fig. 3a). This mutation is predicted to truncate the GDF5 precursor polypeptide by 15 amino acids, deleting the C-terminal two of the seven highly conserved cysteine residues. Restriction analysis with *Xba* I indicated that this mutation was present in all three affected individuals available for analysis, but was not detected in one unaffected family member available for analysis and 50 control individuals (data not shown). In the proband of family 2 (III-3 in Fig. 1b), we sequenced both the *NOG* and *GDF5* genes. We found a novel heterozygous missense mutation, c.1118T > G (p.L373R), substituting an arginine (R) for the highly conserved leucine (L) at amino acid residue 373 in the GDF5 prodomain (Fig. 3b). This substitution created a new *Msp* I restriction site in the mutant allele. Restriction analysis in available family members (five affected and three unaffected) with use of the *Msp* I enzyme confirmed the cosegregation of the mutation with the limb phenotype in family 2 (data not shown). No unaffected individual in family 2 or control individual had the mutation. We did not find any change in the *NOG* gene.

Detection of secreted mature GDF5 protein

To determine the effects of the two novel mutations on dimer formation and secretion of GDF5, we inserted a c-myc epitope tag into the mature domain of the wild-type and mutant GDF5 precursor polypeptides and expressed

them in COS-7 cells using a retroviral construct. We first confirmed efficient transfer of proteins concentrated from the cell supernatant by staining the blots in Ponceau red solution (Fig. 4a). In supernatant from cells transfected with construct expressing the wild-type or the p.L373R mutant GDF5 polypeptide, both of the full-length dimer (pro-GDF5) and the mature dimer of 28 kD (mature GDF5) could be recognized by a monoclonal anti-c-Myc antibody (Fig. 4b). In contrast, these dimers were not detected in the supernatant from cells transfected with the control vector or the construct expressing the p.Y487X mutant GDF5 polypeptide (Fig. 4b). Total cell lysate was also included in the blots, and the GDF5 monomer (mono-GDF5) was confirmed to be present in the lysate of cells transfected with all wild-type and mutant GDF5-containing constructs (Fig. 4b).

Discussion

To date, 14 pathogenic mutations in the *GDF5* gene have been identified in patients affected with BDC (Polinkovsky et al. 1997; Everman et al. 2002; Galjaard et al. 2001; Savarirayan et al. 2003; Schwabe et al. 2004; Holder-Espinasse et al. 2004). These include nine frameshift mutations, four missense mutations and one nonsense mutation. In a family with typical BDC, Polinkovsky and colleagues found the first nonsense *GDF5* mutation, c.901C > T (p.R301X), in the prodomain (Polinkovsky et al. 1997), which is the only nonsense mutation identified so far in all *GDF5*-associated skeletal dysplasia (Seemann et al. 2005; Polinkovsky et al. 1997; Faiyaz-Ul-Haque et al. 2002; Thomas et al. 1996, 1997; Dawson et al. 2006; Wang et al. 2006; Everman et al. 2002; Galjaard et al. 2001; Savarirayan et al. 2003; Schwabe et al. 2004; Holder-Espinasse et al. 2004). In the large Chinese family 1 we studied, a novel nonsense mutation in the active mature domain, c.1461T > G (p.Y487X), was found, representing the first disease-causing nonsense *GDF5* mutation in the mature domain. Based on the fact that most BDC-causing mutations are frameshift mutations deleting the active mature domain, or missense mutations in the mature domain producing abnormal disulfide bonds, functional haploinsufficiency of the *GDF5* gene has been suggested as the mechanism underlying the molecular pathogenesis of BDC (Everman et al. 2002). The c.1461T > G (p.Y487X) mutation was predicted to encode a truncated protein lacking two C-terminal cysteine residues. In COS-7 cells expressing the p.Y487X GDF5 precursor polypeptide, the mature dimer could not be detected in the supernatant. Taken together, our results provided further supportive evidence for *GDF5* haploinsufficiency in BDC.

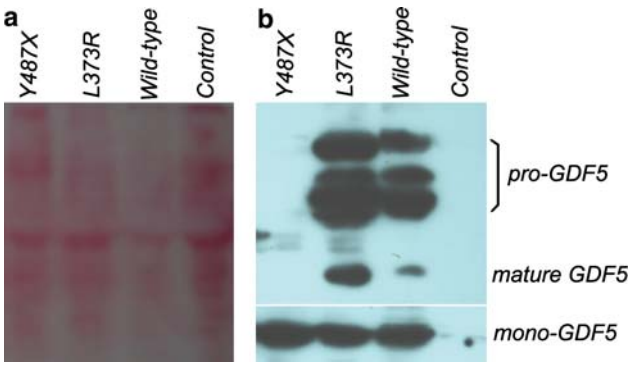

Fig. 4 Dimer formation and secretion of the wild-type and mutant GDF5 proteins. **a** Ponceau red-stained western blot demonstrating efficient transfer of proteins from supernatant samples. **b** Western blot analysis of supernatant (*top*) and lysate (*bottom*) from the wild-type and mutant GDF5 expressing COS-7 cells. Secreted mature dimer (28 kD) and full-length dimer are indicated as mature GDF5 and pro-GDF5, respectively. The monomer detected in lysate is indicated as mono-GDF5

Structural and mutagenesis studies have indicated the importance of the seven highly conserved cysteine residues in the overall structure and ultimate function of the active mature GDF5 molecule (Venkataraman et al. 1995; Griffith et al. 1996; Amatayakul-Chantler et al. 1994; Brunner et al. 1992). This was further supported by the finding of two pathogenic *GDF5* mutations: p.C400Y in Grebe type chondrodysplasia and p.C498S in BDC (Thomas et al. 1997; Everman et al. 2002). In a family with Hunter-Thompson-type acromesomelic dysplasia, an autosomal recessive disease caused by *GDF5* mutations, Thomas and colleagues found a homozygous 22-bp insertion in affected individuals (Thomas et al. 1996). This insertion occurred in the mature domain at a site 11 amino acids after the third cysteine residue, resulting in an altered reading frame that encoded 43 amino acids, including two cysteines. Therefore, this insertion mutation was predicted to produce a mutant GDF5 precursor polypeptide of 486 amino acids with five C-terminal cysteine residues (Thomas et al. 1996). Interestingly, the c.1461T > G (p.Y487X) mutation we identified would also lead to a mutant GDF5 precursor polypeptide with the same total number of 486 residues and five C-terminal cysteine residues instead of seven. While heterozygous carriers of the loss-of-function 22-bp insertion were normal (Thomas et al. 1996), heterozygotes carrying the nonsense mutation in family 1 displayed typical BDC. Nonpenetrant carriers of a *GDF5* insertion mutation had been observed in unrelated families with BDC (Everman et al. 2002). This has led to the suggestion that BDC-causing mutations decrease GDF5 activities just below the biological threshold required for normal limb development and that the variable expression and non-penetrance in BDC can both be influenced by the modifier loci, environmental factors and/or stochastic events (Everman et al. 2002). The absence of limb phenotype in

the carriers of the 22-bp insertion might be due to a mutant GDF5 having some remaining residual activity which is over the biological threshold (Thomas et al. 1996).

Palmoplantar keratoderma consists of a group of heterogeneous genetic diseases. It was not previously reported in any type of the GDF5-associated skeletal dysplasias. In the above-mentioned family 1, a mother and her son (III-11 and IV-6 in Fig. 1a) were also diagnosed with severe palmoplantar keratoderma. It is most likely that BDC and palmoplantar keratoderma in these two affected family members originated from independent mutations of different genes.

The identification of the two missense mutations, p.R438L and p.E491K, in the active mature domain of GDF5 by two independent groups in families with distinct ethnic backgrounds established *GDF5* as the second locus for SYM1 (Seemann et al. 2005; Wang et al. 2006). In the Chinese family with six members affected with typical SYM1, we found the novel c.1118T > G (p.L373R) missense mutation. This mutation was shown to cosegregate perfectly with the limb phenotype in the family. It was not detectable in unaffected family members or in 50 unrelated and ethnically matched control individuals. Leucine at residue 373 in the prodomain is invariant in all GDF5 with amino acid sequences available in the NCBI database. In addition, our linkage and sequence analysis excluded the *NOG* gene and found evidence for linkage to the *GDF5* gene (data not shown). These results, taken together, suggested strongly the pathogenicity of the novel missense *GDF5* mutation in SYM1. No mutation in the prodomain of GDF5 has been reported in familial or sporadic cases of SYM1 (Seemann et al. 2005; Wang et al. 2006). The c.1118T > G (p.L373R) missense mutation might be the first SYM1-associated *GDF5* mutation in its prodomain.

In contrast to various BDC-causing mutations, the SYM1-associated *GDF5* missense mutations in the active mature domain have been suggested to be gain-of-function (Seemann et al. 2005). The p.R438L mutation was first found in an American family with SYM1 and recently was also identified in an Ashkenazi Jewish family with multiple synostoses syndrome type 1 (Seemann et al. 2005). In functional studies using chicken limb bud micromass culture, the p.R438L mutant GDF5 showed increased biological activity, as compared to the wild type. Biosensor interaction analyses indicated that the mutant had acquired BMP2-like properties with increased binding to BMP receptor type 1A (Seemann et al. 2005). The p.E491K mutation recently reported in Chinese SYM1 cases might also act in a similar way (Wang et al. 2006). Recently, Dawson and colleagues observed that cells expressing the p.R438L mutant GDF5 showed normal dimer formation and secretion (Dawson et al. 2006). Similarly, we did not find significant difference in dimer formation and secretion between COS-7 cells expressing the wild-type and the p.L373R mutant GDF5. Further studies on the p.L373R GDF5 mutant would add to our understanding of the functional interaction between GDF5 prodomain and mature domain, and of the molecular pathogenesis of various *GDF5* mutations.

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