P63 mutations are not a major cause of non-syndromic split hand/foot malformation

X J de Mollerat, D B Everman, C T Morgan, K B Clarkson, R C Rogers, R S Colby, A S Aylsworth, J M Graham Jr, R E Stevenson, C E Schwartz

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Ectrodactyly or split hand/foot malformation (SHFM) is a human limb malformation characterised by underdevelopment or absence of the central digital rays and variable fusion of the remaining digits (MIM 183600). This condition occurs in 1 in 8500–25 000 newborns^{1–4} and is usually inherited in an autosomal dominant manner, although a family with an X linked form has also been reported.⁵ At least five loci are responsible for this condition in humans: SHFM1 on 7q21.3–q22.1,^{6,7} SHFM2 on Xq26,⁸ SHFM3 on 10q24,^{9,10} SHFM4 on 3q27,¹¹ and SHFM5 on 2q31.^{12,13}

SHFM is clinically heterogeneous, presenting in either non-syndromic or syndromic forms. The non-syndromic form of SHFM can be isolated (type I) or associated with long bone deficiency (type II).¹⁴ The latter disorder has several names, including cleft hand with absent tibia and SHFM with long bone deficiency (SHFLD (MIM 119100)). Patients with SHFLD have SHFM in association with underdevelopment or absence of one or more long bones, most commonly the tibia.^{14–18} As with other forms of SHFM, the pattern of limb deficiency in SHFLD may be widely variable, even among the limbs of an affected subject. This condition preferentially affects males (M:F 1.6:1), right sided limbs, and lower limbs.¹⁶ Other skeletal findings in SHFLD include hypoplastic hallux, club foot, polydactyly, and bifurcation of the distal femur.¹⁵ Although ectodermal dysplasia^{19,20} and other findings have been seen in some patients with SHFLD, no consistent pattern of non-skeletal anomalies has been observed.

The prototypical syndromic form of SHFM is the EEC syndrome (ectrodactyly, ectodermal dysplasia, and cleft lip/palate) (MIM 129900).²¹ The EEC phenotype includes SHFM, orofacial clefts, and abnormalities of the skin, teeth, hair, nails, lacrimal ducts, and mammary glands.²¹ Interestingly, penetrance in SHFLD (66%) is much lower than in isolated SHFM (96%) or EEC syndrome (93–98%).^{14,21}

Mutations in the *P63* gene, a homologue of the tumour suppressor gene *P53*, have been shown to cause the EEC phenotype at the SHFM4 locus on 3q27.¹¹ This gene spans 65 kb, contains 15 exons, and encodes six different protein isoforms using alternative splicing and two translational start sites.²² *P63* has four major protein domains: a transactivation domain (TA), a DNA binding domain (DBD), an oligomerisation domain (OD), and a sterile alpha motif (SAM) responsible for protein-protein interaction.^{22,23} In situ hybridisation studies in mice and chicken have shown that *P63* is highly expressed in the apical ectodermal ridge (AER) of the limb buds, interdigital tissues, epithelium of branchial arches, and feather buds.^{24–26} This pattern of expression correlates with the phenotype of *P63* knockout mice, which fail to differentiate the AER and have an EEC-like phenotype including severe limb truncation, absence of ectodermal tissues, and craniofacial abnormalities.^{24,25} In humans, previous studies have shown that 93% (42/45) of EEC patients have mutations in *P63*.^{11,27,28} All mutations causing EEC syndrome have been found within the DNA binding domain (encoded by exons 5 to 10), with the exception of one frameshift mutation in exon 13. In contrast,

Key points

- Missense mutations in the DNA binding domain of *P63* were detected in seven out of 10 patients with EEC syndrome. Two of these mutations have not been described previously.
- The three EEC patients without mutations in *P63* had associated abnormalities of the central nervous system. One patient had hydrocephalus and the other two had colobomas, microphthalmia, and microcephaly.
- We have screened 24 other SHFM patients for *P63* mutations: six with isolated SHFM, 16 with SHFM associated with long bone deficiency (SHFLD), one with Karsch-Neugebauer syndrome (MIM 183800), and one with SHFM, seizures, and mental retardation.
- SSCP analysis of the entire coding region and the flanking intronic sequence of *P63* detected no mutations in the 24 SHFM patients.
- These observations support a major causative role for *P63* in EEC syndrome but not in non-syndromic SHFM.

only 16% (6/37) of non-syndromic SHFM patients have mutations in *P63*, including four in the DNA binding domain that have not been found in EEC patients.^{27,28}

The objective of our study was to expand on these findings through mutational analysis of *P63* in a large number of additional patients with both syndromic and non-syndromic SHFM, including those with associated long bone deficiency.

METHODS

Subjects

All patients were ascertained for study based upon the presence of SHFM in one or more limbs. Informed consent was obtained before participation, and these studies were approved by the Institutional Review Board of Self Memorial Hospital (Greenwood, SC). All patients were examined by a clinical geneticist. A diagnosis of EEC syndrome was made if at least two of the following features were present: (1) SHFM, (2) ectodermal dysplasia affecting the hair, nails, teeth, lacrimal ducts, sweat glands, or mammary glands, and (3) clefting of the lip and/or palate. Classification of cases as syndromic or non-syndromic SHFM was based on the presence or absence of extraskeletal abnormalities. Patients with isolated limb defects consistent with SHFM were classified as having non-syndromic SHFM type I according to the classification of Zlotogora.⁴ Patients with SHFM associated with hypoplasia or absence of one or more long bones (tibia, fibula, femur, radius, ulna, humerus) were classified as having non-syndromic SHFM type II, also known as SHFM with long bone deficiency (SHFLD).¹⁴

Table 1 Clinical features and *P63* mutation screening of patients with EEC syndrome

Patient	Ectodermal features*	Limbs†		Clefting‡	Other features	Nucleotide changes	Amino acid changes
		Hands	Feet				
RG	H, La, Ni	C, S, O	T	–	Anterior hair whorl	G728T	R204L
ALT	H, T	C, S, O	C	–		G728A	R204Q
RB	H, Ni	C, S	–	L and P	Hearing loss	G953A	R279H
SH	H, T	C, O	C, S, O	L	Blindness, seizures, MR§	G1028C	R304P
ED	H, T, N, La	C	T, S	L and P		G1028A	R304Q
BH	H, La, N, T	C	C	L and P		G1028A	R304Q
JR	H, N	C, S	C, S	L and P		C1027T	R304W
SN	H, Ni	O, S	C, O	–	Anterior hair whorl, hydrocephalus	None	–
LA	H, La, T	O, M	O, M	L and P	Microcephaly, microphthalmia coloboma	None	–
WJ	H, Ni	C, O	C, O	L	Microcephaly, microphthalmia	None	–

*H=sparse hair, La=lacrimal duct stenosis/atresia, N=nail hypoplasia, T=anodontia or hypodontia, Ni=hypoplastic/inverted nipples. †C=cleft, S=syndactyly, O=oligodactyly, M=monodactyly, T=toe hypoplasia. ‡L=lip, P=palate. §These features are probably attributable to an episode of meningitis that she experienced at the age of 10 months.

Clinical phenotypes

EEC patients

All 10 patients had at least two major findings of EEC syndrome. One case, ALT, was familial with an affected mother and two affected sons. Two patients also had colobomatous microphthalmia and microcephaly, one had hydrocephalus, and another had seizures, mental retardation, and blindness (table 1).

SHFM patients

Among the 24 SHFM patients, six sporadic cases had SHFM while 16 patients, seven familial and nine sporadic, had SHFM associated with long bone deficiency (SHFLD) (SHFM type II). An additional two sporadic cases had syndromic SHFM, including one with nystagmus (Karsch-Neugebauer syndrome (MIM183800)) and one with seizures and mental retardation (table 2).

DNA extraction and SSCP analysis

DNA from peripheral blood leucocytes was extracted using a high salt precipitation procedure.³¹ Mutation analysis was performed using intronic primers that were suitable for amplification of exons 1 to 15 of *P63*.^{11–27} PCR was performed in a total volume of 10 µl, using 50 ng genomic DNA. The reactions were carried out at a final concentration of 50 mmol/l KCl, 1.5 mmol/l MgCl₂, 1 µmol/l of each primer, 50 µmol/l dNTPs, 1 µCi α³²P dCTP, and 0.5 units of *Taq* DNA polymerase (Sigma, St Louis, MO, USA). PCR conditions were as follows: one cycle at 95°C for four minutes; 20 cycles at 95°C for 30 seconds, annealing at 65°C with a decrement of 0.5°C/cycle for 30 seconds, and elongation at 72°C for 30 seconds; 15 cycles at 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds; and one cycle at 72°C for five minutes. Amplification was carried out in an MJ Research thermocycler (MJ PTC-2000); 5 µl of the ³²P labelled PCR product was denatured in loading buffer (95% formamide, 10 mmol/l NaOH, 0.25% bromophenol blue, 0.25% xylene cyanol), and analysed on a 0.5 × MDE (FMC, Rockland, ME, USA) gel at 8 W for 18 hours. The radioactive signal was visualised using Biomax MS film (Kodak, Rochester, NY) after two hours of exposure at –80°C.

Sequencing

Both strands of the PCR products generated from the patients and a normal control DNA were sequenced using the ThermoSequenase CyTM5.5 Dye Terminator kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) with exon specific primers covering 30 to 50 bp of flanking intronic sequences.^{11–27} Electrophoresis and analysis were performed on an Automated Laser Fluorescence (ALF) DNA sequencer after purification with Autoseq columns (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Mutation confirmation by restriction digest

The point mutations G728T and G728A removed a *Ban*I site in exon 5. The 291 bp PCR product corresponding to exon 5 was digested with *Ban*I, producing two fragments for controls (194 bp and 97 bp) and three fragments for each patient (291 bp, 194 bp, and 97 bp). The point mutation G953A removed an *Acc*I site in exon 7. The PCR product of 253 bp corresponding to exon 7 was digested with *Acc*I, producing two fragments for controls (143 bp and 110 bp) and three fragments for the patient (253 bp, 143 bp, and 110 bp).

The point mutations G1028C, G1028A, and C1027T removed an *Msp*I site in exon 8. The 260 bp PCR product corresponding to exon 8 was digested with *Msp*I, producing two fragments for controls (157 bp and 103 bp) and three fragments for each patient (260 bp, 157 bp, and 103 bp). All fragments were resolved by electrophoresis on a 2% Seakem[®] LE agarose gel (BMA, Rockland, ME, USA).

Polymorphism studies

Base changes were classified as polymorphisms if they fulfilled at least one of the following criteria: (1) the change was also present in the normal white subject who was used as our sequencing control, or (2) the change was not present in an affected parent.

RESULTS

P63 mutation analysis in EEC patients

Screening of the DNA binding domain (exons 5 to 10) of *P63* showed seven missense mutations in seven out of 10 unrelated patients with EEC syndrome, including two previously unreported mutations found in patients RG and SH. Patient RG has symmetrical split hand malformations with syndactyly of the thumb (digit 1) and the index finger (digit 2) with separate nails, absent digit 3 with a shallow cleft of the hand, and separate digits 4 and 5. Her left foot has a short second toe with a hyperconvex nail and partial syndactyly of the first and second toes. She also has inverted nipples, thin hair, lacrimal duct stenosis, and a short nasal septum but no clefting of the lip and/or palate (fig 1A).

Patient RG had an abnormal SSCP pattern in exon 5 (fig 2A). Sequence analysis showed a G to T transversion at position 728 (G728T), which predicts an amino acid substitution, arginine for leucine, at position 204 (R204L) (fig 2B). The G728T alteration removes a *Ban*I site and the restriction digest confirmed the mutation (fig 2C). This patient is a sporadic case and genetic analysis of both parents showed the mutation to be de novo (fig 2C). The mutation was not detected in 192 control chromosomes.

Patient SH has bilateral split hand malformations with absence of digits 2 and 3. Her right foot has a wide cleft with an absent second toe and syndactyly of the remaining toes and

Table 2 Clinical features of SHFM patients

SHFM type	Patient	Limbs*		Long bone deficiency	Family history	Other findings
		Hands	Feet			
SHFM type II	WB†	C	O, H	Tibial aplasia	Mother has split hand malformation; 2 maternal 1st cousins twice removed were reported to have limb deficiencies similar to proband	
SHFM type II	FL†	C, M	O	Tibial aplasia, humerus hypoplasia, forearm a/hypoplasia	Twin brother and mother have phenotype similar to proband; extensive family history of SHFM type II in autosomal dominant pattern with incomplete penetrance	
SHFM type II	JP†	C, S, M	S, H	Tibial aplasia, forearm a/hypoplasia	Mother has finger syndactyly and toe hypoplasia; maternal great aunt, uncles, and 2nd cousins reported to have variable limb deficiencies	
SHFM type II	LH†‡	C, S	O, S	Tibial hypoplasia	Extensive family history of SHFM type II in autosomal dominant pattern with incomplete penetrance	Preauricular tags in proband
SHFM type II	FW	N	C, S	None	Mother has tibial a/hypoplasia with split hand malformation and monodactylous feet; maternal aunt and grandfather have split foot malformations	Small, malformed ears in proband, mother, and aunt; hearing loss in aunt and grandfather
SHFM type II	EH†	S, Cl	H, N	Tibial aplasia	Great grandmother had split hand malformation; several other relatives reported to have variable limb deficiencies	Distal duplication of femur in proband
SHFM type II	RC	C	C	Left tibia aplasia	Familial	
SHFM type II	ACR	O, S, M	S	Forearm hypoplasia	Sporadic	Hearing loss
SHFM type II	AM	M	O, S	Tibial aplasia, femoral hypoplasia, ulnar v radial aplasia	Sporadic	
SHFM type II	TS	N	O, S, H	Tibial and fibular hypoplasia	Sporadic	Cutaneous haemangioma
SHFM type II	JL†	O, S	O, S	Forearm hypoplasia, femoral hypoplasia, fibular aplasia	Sporadic	
SHFM type II	CN†	C, S	N	Ulnar hypoplasia	Sporadic	
SHFM type II	AS†	C, M	N	Forearm a/hypoplasia	Sporadic	
SHFM type II	LL†	N	C, M	Tibial v fibular aplasia	Sporadic	
SHFM type II	ME†	N	O, H	Tibial v fibular hypoplasia	Sporadic	
SHFM type II	JS	N	O, S, H	Tibial hypoplasia	Sporadic	Facial asymmetry
SHFM type I	KI	M	N	None	Mother has central toe hypoplasia	
SHFM type I	RC	Na	C, Na	None	Sporadic	
SHFM type I	RM	N	C, S	None	Sporadic	Short nasal septum, dry skin
SHFM type I	CD	S, Cl, P	C	None	Sporadic	Triphalangeal thumb, bifid thumb
SHFM type I	ZO	O, S	C	None	Sporadic; 4th degree relative with isolated cleft palate	Sparse hair and eyebrows
SHFM type I	KC	C	C, H	None	Sporadic	
SHFM-Syn	KO	C	S	None	Sporadic	Childhood seizures, mental retardation
SHFM-KNS	NG	S, P	C	None	Sporadic	Nystagmus, developmental delay, Karsch-Neugebauer syndrome

*N=no abnormalities observed, O=oligodactyly, C=oligodactyly with clefting, Cl=clefting without oligodactyly, S=syndactyly, M=monodactyly, H=hypoplastic digits, P=polydactyly, Na=nail hypoplasia. †Previously described.²⁹

‡Clinical phenotype of the family was previously described.³⁰

Figure 1 Photographs of patients RG and SH. (A) Patient RG with a mild variant of EEC syndrome caused by the mutation R204L. She has thin hair with an anterior hair whorl, lacrimal duct stenosis, bilateral split hand malformations, and a short second toe on the left foot. (B) Patient SH with EEC syndrome caused by the R304P mutation. She is blind in the left eye and has corneal opacity of the right eye. She has cleft palate, anodontia, bilateral split hand malformations, a cleft right foot, and syndactyly of the toes on her left foot.

her left foot has syndactyly of toes 1/2 and 4/5. She also has cleft palate, anodontia, and macrostomia. She is severely mentally retarded (IQ=8) and has a seizure disorder. She is blind in the left eye and has corneal opacity of the right eye (fig 1B). She had meningitis at the age of 10 months.

Patient SH had an altered SSCP pattern of exon 8 (fig 3A). Sequence analysis showed a G to C transversion at position 1028 (G1028C), which predicts an amino acid substitution, arginine to proline, at position 304 (R304P) (fig 3B). This mutation results in the loss of an *MspI* site in the patient, which made it possible to confirm the mutation by restriction digest (fig 3C). This patient is a sporadic case whose parents were not available for genetic testing. The mutation was not detected in 192 control chromosomes.

Five other patients with typical EEC syndrome also had *P63* mutations. All have been reported previously as disease causing mutations in EEC syndrome.¹¹⁻²⁷ These include a G to A transition at nucleotide 728 (G728A) which predicts an arginine to glutamine substitution at position 204 (R204Q); a G to A transition at position 953 (G953A) which predicts an

arginine to histidine change at position 279 (R279H); a G to A transition at position 1028 (G1028A) which predicts an arginine to glutamine change at position 304 (R304Q) (two patients); and a C to T transition at position 1027 (C1027T) which predicts an arginine to tryptophan change at position 304 (R304W) (table 1).

In the three EEC patients lacking mutations in the DNA binding domain of *P63*, the analysis was extended to the entire coding region and flanking intronic sequences and no mutations were detected. Interestingly, all three patients had central nervous system abnormalities. Two of these patients had colobomatous microphthalmia and microcephaly, and the third patient had hydrocephalus.

P63 mutation analysis in SHFM patients

SSCP analysis of the entire coding region and flanking intronic sequences of *P63* did not detect any mutations in our panel of 24 patients with various forms of SHFM (table 2). However, six different polymorphisms were discovered. In one patient with SHFM type I, a 2 bp deletion was found in intron

Figure 2 Mutation analysis of exon 5 of *P63* in patient RG. (A) SSCP analysis using intronic primers flanking exon 5. Lane 1: control; lane 2: mother; lane 3: patient RG. (B) Sequencing of exon 5 from a control (upper fluorogram) and patient RG (lower fluorogram). The G to T change is seen as a mixed sequence signal (arrows). (C) The G to T change destroys a *BanI* site. Digestion of a 291 bp PCR fragment encompassing exon 5 normally produces fragments of 194 bp and 97 bp. The mutated allele is not cut by *BanI* and migrates as an undigested 291 bp fragment present only in patient RG (lane 2) and in neither of the unaffected parents (lanes 1 and 3) nor in a control (lane 4).

Figure 3 Mutation analysis of exon 8 of *P63* in patient SH. (A) SSCP analysis using intronic primers flanking exon 8. Lane 1: patient SH; lanes 2, 3, 4: controls. (B) Sequencing of exon 8 from a control (upper fluorogram) and patient SH (lower fluorogram). The G to C change is seen as a mixed sequence signal (arrows). (C) The G to C change destroys an *MspI* site. Digestion of a 260 bp PCR fragment encompassing exon 8 normally produces fragments of 157 bp and 103 bp. An undigested 260 bp fragment is observed in patient SH (lane 2) but not in controls (lanes 1 and 3).

2, 8 bp from the splice acceptor site (IVS29-8delAT). The affected mother did not carry this change and RT-PCR analysis using primers located in exons 1 and 4 respectively did not show any aberrant splice forms in the patient carrying the deletion (data not shown). Two SHFM type II patients and one SHFM type I patient had a T to G change 35 bp after the intron 5 splice acceptor site (IVS5+35T>G). This change was seen in a control and an unaffected family member of one patient. A sporadic SHFM type I and a type II patient both had a C to T change at position 1198 (C1198T) in exon 6. However, this change does not alter the amino acid (L261L) and was also observed in a familial SHFM3 patient linked to chromosome 10q24. It is likely this substitution is a polymorphism. The other polymorphic changes found in patients and one control were an A to T change in intron 8 at position -22 (IVS8-22A>T) and a G to C change in intron 10 at positions +40 and +41 (IVS10+40G>C, IVS10+41G>C).

DISCUSSION

We detected seven missense mutations in *P63* in seven out of 10 patients with EEC syndrome. The mutations involved codons for arginine residues at positions 204 (R204Q, R204L), 279 (R279H), and 304 (two R304Q, R304W, R304P). R204, R279, and R304 are considered three of the five mutational hot spots in *P63*²⁷ and account for 65% (34/52) of all the *P63* mutations found in patients with EEC syndrome^{11 27 28} (present data).

In the three EEC patients without mutations in the DNA binding of *P63*, we extended the analysis to the entire coding region and flanking intronic sequences but no mutations were detected. Interestingly, these three patients also had abnormalities of the central nervous system. One patient had

hydrocephalus along with typical split foot malformations but atypical changes in the hands (missing thumb, 3-4 syndactyly). This patient also had thin hair, lacrimal duct stenosis, and hypoplastic nipples. The other two patients had typical EEC features in association with colobomatous microphthalmia and microcephaly. It is possible that a large deletion or inversion may have disrupted *P63* and another nearby gene involved in the development of the central nervous system (CNS). This might be the case in the two patients with eye defects since a locus for eye formation was mapped to 3q27.³²

The lack of other reported patients with hydrocephalus and SHFM makes it difficult to propose an alternative candidate locus for this phenotype. However, several other loci have been associated with phenotypes including EEC-like features and eye defects. Microcephaly with and without colobomas has been observed in several patients with syndromic SHFM and visible deletions or rearrangements involving the SHFM1 locus on 7q21.3-q22.1.⁷ Thus, it is possible that these three patients could have alterations at this locus. However, no cytogenetically visible rearrangements were detected in these patients.

Deletions of chromosome 2q31-33 have also been observed in patients with SHFM and multiple anomalies including cleft palate, growth retardation, microcephaly, retinal coloboma, and microphthalmia.^{13 34} These observations suggest that another locus for an EEC-like syndrome associated with microcephaly, coloboma, and microphthalmia is located on 2q31-33.

One patient with SHFM, cleft lip and palate, microphthalmia, and mental retardation was previously reported.³⁵ This patient carried a chromosome 6;13 translocation, suggesting another possible locus for this condition on either 6q21 or 13q12.³⁵

Since both of our patients with eye defects and EEC features were female, they could represent variants of the X linked male lethal Goltz-Gorlin (focal dermal hypoplasia) syndrome (MIM 305600). This condition shares clinical features with EEC syndrome and also includes a/microphthalmia and coloboma. However, our patients did not have the skin anomalies characteristic of this syndrome.³³

Two of the missense mutations observed in our study (R204L and R304P) have not been described previously. Patient RG, with the R204L mutation, has EEC syndrome without cleft lip/palate (fig 1A). R204W and R204Q substitutions have been reported, and both substitute polar amino acids for arginine, a basic amino acid. It has been suggested that these substitutions create extensive structural rearrangements affecting the DNA binding activity of P63.¹¹ Leucine is also a polar amino acid, and a substitution of leucine for arginine is likely to have a similar effect on the DNA binding activity of P63.

Patient SH, with the R304P mutation, has EEC syndrome associated with mental retardation, blindness, and a seizure disorder (fig 1B). Proline contains a cyclic ring, which creates a fixed kink in a polypeptide chain. Therefore, substitution of proline for arginine should cause a more drastic effect on the DNA binding activity of P63 than the other R304 mutations reported (R304Q, R304W). While our finding raises the possibility of a relationship between this mutation and the patient's mental retardation, blindness, and seizures, these findings are more likely attributable to an episode of meningitis that she experienced at the age of 10 months. Identification of additional patients with the same mutation would thus be helpful to confirm or exclude a possible role of P63 in brain development and function.

Using SSCP analysis, we have screened 22 non-syndromic SHFM patients and two syndromic SHFM patients for mutations in P63. Sixteen of those with non-syndromic SHFM had long bone deficiency and six had isolated type I SHFM. The other two patients with syndromic SHFM included one with nystagmus (Karsch-Neugebauer syndrome) and one with seizures and mental retardation. No mutations were detected in the entire coding region and intronic flanking sequences of P63 in our panel of 24 SHFM patients.

In two previous studies, sequencing of the entire coding region and the flanking intronic sequences of P63 showed mutations in 16% (6/37) of non-syndromic SHFM cases studied, including one family with evidence of long bone deficiency.^{27, 28} There are several possible explanations for the discrepancy between these results and our findings. The reported non-syndromic SHFM patients with P63 mutations could represent EEC patients expressing mild ectodermal features. However, extensive clinical investigation in two cases did not show ectodermal defects or clefting that would suggest EEC syndrome.²⁸ Detailed clinical information was not available for the other four non-syndromic SHFM patients.²⁷ Moreover, five out of the six mutations found in these cases differed from those detected in patients with EEC syndrome.

In our study population, 73% (16/22) of non-syndromic SHFM patients had associated long bone deficiency. Previous studies have screened P63 in 37 non-syndromic SHFM patients. However, it is not clear how many of these patients had long bone involvement.^{27, 28} Mutations in P63 may only be present in a small number of patients with non-syndromic SHFM, most of whom do not have long bone deficiency. This would potentially explain why no mutations were found in our non-syndromic SHFM patients. It is also possible that gross DNA rearrangements (insertions, deletions, or inversions) may exist in these patients, and neither SSCP analysis nor direct sequencing of the P63 coding region would have detected these rearrangements. Alternatively, since several loci have been characterised for isolated SHFM,⁵⁻¹³ our data support the hypothesis that P63 is not a major cause of non-syndromic SHFM.

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Authors' affiliations

X J de Mollerat, D B Everman, C T Morgan, K B Clarkson, R C Rogers, R S Colby, R E Stevenson, C E Schwartz, Center for Molecular Studies, J C Self Research Institute of Human Genetics, Greenwood Genetic Center, Greenwood, South Carolina, USA
X J de Mollerat, D B Everman, R E Stevenson, C E Schwartz, Department of Genetics and Biochemistry, Clemson University, South Carolina, USA
A S Aylsworth, Department of Pediatrics, University of North Carolina, Chapel Hill, North Carolina, USA
J M Graham Jr, Medical Genetics Birth Defects Center, Department of Pediatrics, Cedars Sinai Medical Center, UCLA School of Medicine, Los Angeles, California, USA

Correspondence to: Dr C Schwartz, Center for Molecular Studies, J C Self Research Institute, One Gregor Mendel Circle, Greenwood, SC 29646, USA; schwartz@ggc.org

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