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p63 Gene Mutations in EEC Syndrome, Limb-Mammary Syndrome, and Isolated Split Hand–Split Foot Malformation Suggest a Genotype-Phenotype Correlation

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_p63_ mutations have been associated with EEC syndrome (ectrodactyly, ectodermal dysplasia, and cleft lip/palate), as well as with nonsyndromic split hand–split foot malformation (SHFM). We performed _p63_ mutation analysis in a sample of 43 individuals and families affected with EEC syndrome, in 35 individuals affected with SHFM, and in three families with the EEC-like condition limb-mammary syndrome (LMS), which is characterized by ectrodactyly, cleft palate, and mammary-gland abnormalities. The results differed for these three conditions. _p63_ gene mutations were detected in almost all (40/43) individuals affected with EEC syndrome. Apart from a frameshift mutation in exon 13, all other EEC mutations were missense, predominantly involving codons 204, 227, 279, 280, and 304. In contrast, _p63_ mutations were detected in only a small proportion (4/35) of patients with isolated SHFM. _p63_ mutations in SHFM included three novel mutations: a missense mutation (K193E), a nonsense mutation (Q634X), and a mutation in the 3′ splice site for exon 5. The fourth SHFM mutation (R280H) in this series was also found in a patient with classical EEC syndrome, suggesting partial overlap between the EEC and SHFM mutational spectra. The original family with LMS (van Bokhoven et al. 1999) had no detectable _p63_ mutation, although it clearly localizes to the _p63_ locus in 3q27. In two other small kindreds affected with LMS, frameshift mutations were detected in exons 13 and 14, respectively. The combined data show that _p63_ is the major gene for EEC syndrome, and that it makes a modest contribution to SHFM. There appears to be a genotype-phenotype correlation, in that there is a specific pattern of missense mutations in EEC syndrome that are not generally found in SHFM or LMS.

Introduction

The central reduction defect of the hands and feet known as ectrodactyly, split hand–split foot malformation (SHFM) or lobster-claw, occurs in ~1/18,000 newborns (Czeizel et al. 1993; Evans et al. 1994). On the basis of linkage studies and the analysis of chromosomal abnormalities, three loci have been identified in humans: SHFM1 on 7q21.3-q22.1 (MIM 183600; Scherer et al. 1994; Crackower et al. 1996), SHFM2 on Xq26 (MIM 313350; Faiyaz ul Haque et al. 1993), and SHFM3 on 10q24-q25 (MIM 600095; Nunes et al. 1995; Raas-Rothschild et al. 1996). In almost 40% of the cases, ectrodactyly is associated with other anomalies (Czeizel et al. 1993; Evans et al. 1994). A well-known example

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Table 1

Oligonucleotides and PCR Conditions for *p63* Mutation Analysis

Exon	Primer Sequences		Size of PCR Product (bp)	Conditions	
	Forward	Reverse		Annealing Temperature (°C)	$MgCl_2$ Concentration
1	TC CCG GCT TTA TAT CTA TAT ATA C	GAC ACA TTC ATA ATA CAC AAG GCA C	211	59	2.0
2	TCC ACT TGG GTT TTC ATG ATA GAG	GTA AGC AAT ATT TTG ACC ACC CAC	300	58	2.5
3	GCT TGT TGT TAA CAA CAG CAT G	GAA AAG ACA GGT TTA ACA GAG C	281	59	2.5
3′	CAT ATT GTA AGG GTC TCA GAG G	GAC CGA GAA CCG CAA ATA CG	223	59	1.0
4	GAT CCG TGG CTT CAG CGG	AAG CCC ATC CTT GGA CTT GG	354	58	2.5
5	GTT GGT TCT CTC CTT CCT TTC	GCC CAC AGA ATC TTG ACC TTC	291	57	3.0
6	CCA CCA ACA TCC TGT TCA TGC	GTT CTC TCA AGT CTA CTC AGT CC	267	55	2.5
7	GGG AAG AAC TGA GAA GGA ACA AC	CAG CCA CGA TTT CAC TTT GCC	253	55	2.5
8	GTA GAT CTT CAG GGG ACT TTC	CCA ACA TCA GGA GAA GGA TTC	260	54	2.5
9	GCT TTA GAA GTG TTC CCA GG	ACA CCT CCT TTC CCA TTG TC	237	55	2.5
10	TGA GGA TTG ACC ACA CTT CTA AC	CAT CAA TCA CCC TAT TGC TGA TC	287	61	2.0
11	TGA NCA TCA TTT CCA TGT TTG TC	TCA CAG AGT CTT GTC CTA AGC	254	57	3.0
12	GGA CTA TAA CAG TAT CCG CCC	CAA GAT GGA CCA CTG GGA TG	294	60	2.0
13	CTT ATC TCG CCA ATG CAG TTG G	AAC TAC AAG GCG GTT GTC ATC AG	240	55	2.5
14	GGG AAT GAT AGG ATG CTG TGG	AAG ATT AAG CAG GAG TGC TT	449	54	2.0
15	GAT GAA GTC CTA GGC CTT C	GGA AAT ACA ACA CAC ACA CT	205	55	1.0

is EEC syndrome, which comprises ectrodactyly, ectodermal dysplasia, and cleft lip with or without cleft palate (MIM 129900 and 602077). EEC syndrome has an autosomal dominant mode of inheritance with highly variable expression and reduced penetrance. Ectodermal dysplasia in EEC syndrome affects the skin, hair, nails, sweat glands, and teeth (Rodini and Richieri-Costa 1990; Roelfsema and Cobben 1996). Other symptoms are lacrimal-duct abnormalities, urogenital problems, conductive hearing loss, facial dysmorphism, chronic/recurrent respiratory infections, and developmental delay (Gorlin et al. 1990). We recently mapped the genetic defect in EEC syndrome and in an EEC-like condition called "limb-mammary syndrome" (LMS; MIM 603543) to chromosome 3q27 (Celli et al. 1999; van Bokhoven et al. 1999). The differentiation of LMS and EEC syndrome is based on three findings. (1) Mammary-gland hypoplasia and nipple hypoplasia are consistent features in LMS and are observed occasionally in EEC syndrome. (2) Patients with LMS have no hair and skin defects. (3) Patients with EEC syndrome have cleft lip with or without cleft palate (CLP), whereas patients with LMS have cleft palate only (CP). The distinction between CLP and CP is most relevant, because the affected structures—the primary palate and the secondary palate, respectively—develop independently of each other (Ferguson 1988; Murray 1995). The *p63* gene mapped to the critical LMS/EEC region on 3q27 (Osada et al. 1998; Senoo et al. 1998; Trink et al. 1998; Yang et al. 1998). Subsequent analysis of the *p63* gene revealed heterozygous mutations in nine unrelated patients with EEC syndrome (Celli et al. 1999). Human phenotypes caused by mutations in the *p63* gene are inherited in an autosomal dominant pattern. These human phenotypes resemble that of *p63*-knockout mice (Mills et al. 1999; Yang et al. 1999). More recently, different missense mutations were described in two large families with SHFM (Ianakiev et al. 2000). We investigated the *p63* gene for mutations in a large group of patients with EEC syndrome, in three individuals and families with the EEC-like LMS, and in 35 patients with SHFM.

Methods

Patients

Patients were enrolled, with informed consent, through clinical genetic centers in the Netherlands, Belgium, the United States, Italy, Israel, Turkey, Austria, Germany, and the United Kingdom. The study was approved by the Nijmegen University Hospital Committee for Research on Human Subjects. All EEC families and isolated patients had at least two of the three main features of the syndrome. These features are (1) an ectodermal dysplasia affecting the skin, hair, nails, teeth, sweat glands, lacrimal ducts, or mammary glands; (2) hand or foot abnormalities consistent with the split hand–split foot spectrum; and (3) cleft lip with or without cleft palate.

LMS fulfills the EEC criteria. However, the phenotype is significantly different from classical EEC syndrome, since the clefting is of the palate alone, the hair and skin are excluded from the ectodermal involvement, and there is consistent mammary-gland and nipple aplasia or hypoplasia (van Bokhoven et al. 1999).

p63 *Mutation Analysis*

Intron-specific primers were designed that are suitable for amplification of exons 1–15 and exon 3′ of the *p63* gene (table 1). PCR reactions were carried out in a 25-μl volume containing 100 ng genomic DNA, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, varying concentrations of $MgCl_2$ (see table 1), 0.2 mM dNTPs, 100 ng of each primer and 1 unit *Taq* polymerase (Gibco-BRL), using the following parameters: 1 min at 94°C, 2 min at annealing temperature, and 1 min at 72°C for 35 cycles (see table 1 for specific annealing temperatures). PCR products were fractionated by electrophoresis in 2% agarose gels and visualized by staining with ethidium bromide. DNA fragments were excised from the gel and were purified with the Qiaquick gel extraction protocol (Qiagen). Sequencing of these fragments was done with the BigDye Terminator chemistry (PE Applied Biosystems) and the use of exon-specific primers. Electrophoresis and analysis were performed on an ABI Prism 3700 (PE Applied Biosystems).

In familial cases, it was verified that the mutation segregated with the phenotype. For sporadic cases, the absence of mutation was tested in both parents, if they were available for these studies. All of the sequence alterations were checked in ⩾50 control individuals, either by allele-specific oligonucleotide (ASO) hybridization, by restriction enzyme digestion of PCR products from the relevant exons, or by direct sequencing. ASO hybridization and washing were performed with primers 5′-ATGAAC(C/G)ACCGTCCAATT-3′ for R279H, 5′-GTGGTGAAG(C/T)GGTGCCCCAC-3′ for R204W, 5′-GTGGTGAAGC(G/A)GTGCCCCAC-3′ for R204Q, as described by Shuber et al. (1993). The position of mutations corresponds to the coding sequence for the original published *TA-p63α* isotype (GenBank accession AF075430) (Yang et al. 1998), which does not include the 39 additional codons for the amino-terminal end that were reported later by Hagiwara et al. (1999) (GenBank accession AF091627).

Exon Trapping

The effect of the 3′ splice-site mutation in intron 4 was investigated by exon trapping. For this, exon 5 and its flanking intron sequences were amplified with primers ET5F (5′-CTG GAG CTC GCC AGT CAA TAT CTC CTG TT-3′) and ET5R (5′-TGG GAT CCC TGA TCT GCC ATC GGA GTG-3′), using genomic DNA from the SHFM patient with the intron 4 splice-site mutation as template. Amplification conditions were as described above. PCR products were digested with *Sst*I and *Bam*HI, for which unique recognitions were contained in the amplification primers. The resulting 187-bp fragments were cloned in the *Sst*I and *Bam*HI sites of pSPL3 (Church et al. 1994). The integrity of the resulting clones, containing either the wild-type sequence or the splice site mutation, was checked by sequence analysis. Cos-1 cells were transfected with either wild-type or mutant construct, and exon trapping was performed according to the manufacturer's conditions (Life Technologies). The resulting RT-PCR products were analyzed by means of electrophoresis on a 1.5% agarose gel. Bands of 364 bp were excised from the gel and were sequenced, by use of primers SD2 and SA4, for the pSPL3-encoded exons (Life Technologies).

FISH Analysis

Metaphase spreads from lymphocytes were prepared using standard procedures, and FISH was performed as described elsewhere (Suijkerbuijk et al. 1991). DNA from YAC clone 889A7 (Celli et al. 1999) was labeled with digoxigenin (Boehringer) and was immunocytochemically detected. Chromosomes were stained with 4′,6-diamidino-2-phenylindole-dihydrochloride (DAPI). Centromere 3–specific staining was obtained with a Cy3-labeled alphoid DNA probe (Waye and Willard 1989).

Results

Mutation Analysis

Comprehensive analysis of the coding region of the *p63* gene included exons 1–15 and the flanking intronic sequences and used the primers listed in table 1. A total of 46 mutations were detected in individuals with EEC, LMS, and SHFM (table 2 and fig. 1), including 9 mutations that have been reported elsewhere (Celli et al. 1999). Evidence that the mutations are indeed causative for these disorders was provided by several observations. (1) The changes are de novo in 27 of the 46 cases. For 16 of the 23 different types of mutations, the occurrence of de novo mutations could be demonstrated. (2) For 10 of the 13 familial cases, additional patients and healthy relatives were available for genetic testing (table 2). Cosegregation of the mutation and phenotype was observed in all 10 of these families. (3) All missense mutations are predicted to affect the DNA-binding capacity of *p63* (Celli et al. 1999; Ianakiev et al. 2000; G. Vriend, P. H. G. Duijf, and H. van Bokhoven, unpublished data). Accordingly, impaired transactivation activity and altered regulation of transactivation were observed for the two mutations that have been tested (C306 R and R304W; Celli et al. 1999 and results not shown). (4) None of the mutations were identified in a cohort of 50 control individuals.

Mutations were detected in 40 unrelated patients and families, of a total of 43 families affected with EEC. This series includes the 9 mutations reported elsewhere (Celli et al. 1999) and 31 not reported elsewhere (table 2 and fig. 1). Missense mutations of codon R204 were present

Table 2

Clinical Features of EEC and LMS Families Tested for *p63* Gene Mutations

Syndrome and Family	No. of Individuals Affected	Ectodermal Features[a]	Mammary Gland[b]	Limb[c]	Clefting	Renal	Additional Features	Mutation	
								Nucleotide	Amino Acid
EEC:									
Q[d]	4	H, T	+	SHFM	−			728G→A	R204Q
AK	1	H, N		Syndactyly, SHFM	CLP		Mandibular prognathism	728G→A	R204Q
DH	2	H, N, T		Syndactyly	−			728G→A	R204Q
EG	1	L, N, T	+	Syndactyly	−			728G→A	R204Q
F[d]	1	H, L, N, T		SHFM	−	+		727C→T	R204W
S[d]	1	H, L, N, S, T		Syndactyly	CLP	+		727C→T	R204W
AA[d]	1	L, N, S, T		Syndactyly	CLP	+		727C→T	R204W
AT	3	H, L, N, S		SHFM	CLP			727C→T	R204W
BB	1	N		SHFM	?		Mild hypoplastic tibia	727C→T	R204W
DE	1	H, L, N, S		SHFM	CLP		Vaginal stenosis	727C→T	R204W
M	6	H, L, N, S, T	+	SHFM	−	+	Micturiction, urethral stenosis, anal stenosis	797G→A	R227Q
O	14	H, L, N, S, T	+	Syndactyly, SHFM	CLP	+	Micturiction, atrophic bladder epithelium, abnormal inner ear	797G→A	R227Q
AS	2	L, T		SHFM	−			797G→A	R227Q
K5440	2	H, L, N		SHFM	CLP			923G→A	C269Y
E[d]	1	H, L, T		Syndactyly, SHFM	CLP			932G→A	S272N
AP	1	S, T		SHFM	CLP			952C→T	R279C
DR	1	H, N, T		SHFM	−			952C→T	R279C
DZ	2	L, S		SHFM	CLP		Recurrent cholesteatoma	952C→T	R279C
N[d]	4	H, L, N, S, T	+	SHFM	CLP		Corneal scarring/vision loss, hernia, mental retardation	953G→A	R279H
AG	1	T		SHFM	−		Mandibular retrognathism	953G→A	R279H
AN	1	L, T		Syndactyly	−		Nails on both sides of fifth finger, micturiction	953G→A	R279H
AX	1	H, L, N, S, T		SHFM	−		Hypohydrosis	953G→A	R279H
BV	1	H, L, N		SHFM	−		Choanal atresia, synblepharon, supernumerary nipple	953-954GC→AA	R279Q

V	11	H, L		Syndactyly, SHFM	CLP		Photophobia	955C→T	R280C
AM	1	L		SHFM	CLP		Mandibular retrognathism	955C→T	R280C
AQ	3	H, L, N, S, T		SHFM	−			955C→T	R280C
DO	1	H, L, S, T		Syndactyly, SHFM	−		Eczema	956G→A	R280H
DX	2	H, N, S, T		SHFM	−		Eczema	955C→A	R280S
L[d]	2	H, L, N, S, T		Syndactyly	CLP	+	Hearing loss	1027C→T	R304W
AJ	1	H, L, N		SHFM	CLP			1027C→T	R304W
Z	1	H, L, N, S, T		Syndactyly, SHFM	CLP	+		1028G→A	R304Q
AE	1	H, L, T		SHFM	CLP			1028G→A	R304Q
AH	1	H, L, N, S, T		SHFM	CLP			1028G→A	R304Q
CN	1	H, L, N, T		SHFM	−		Photophobia, uterine fibroma	1028G→A	R304Q
DY	1	H, L		SHFM	CLP	+		1028G→A	R304Q
H[d]	1	N, S, T		SHFM	−			1033T→C	C306R
U	1	H, L, N, S, T		Syndactyly	−		High palate, hypertelorism, small eyes, recurrent chest infections, eczema	1039T→A	C308S
AZ	1	H, L, N, S, T		SHFM	−			1042C→T	P309S
DF	1	H, L, N, S, T		Syndactyly, SHFM	CLP		Unilateral hydrocele, photophobia, chronic blepharitis, corneal ulcers	1051G→C	D312H
I[d]	1	L, T	+	SHFM	CLP			1689InsA	FS exon13
AC[e]	1	L, N, S, T	+	Syndactyly	CP			None	No mutation
AF[e]	1	N, S, T	+	SHFM	CP			None	No mutation
AO	2	L, T		SHFM	−			None	No mutation
LMS:									
A	29	L, N, T	+	SHFM	CP			None	No mutation
BX	1	T	+	SHFM	CP			1693-1694DelTT	FS exon 13
DW	1	L, N, S	+	SHFM	CP		Anteriorly placed anus, earpits	1860-1861DelAA	FS exon 14

[a] H = sparse hair; L = lacrimal-duct abnormalities; N = nail dystrophy; S = dry skin; T = anodontia/hypodontia.

[b] Mammary-gland hypoplasia and/or nipple aplasia.

[c] Syndactyly = syndactyly or other malformation.

[d] The mutation in these families have been reported elsewhere by Celli et al. (1999).

[e] These patients have been classified as having EEC syndrome, but there is strong phenotypic overlap with LMS. See text for details.

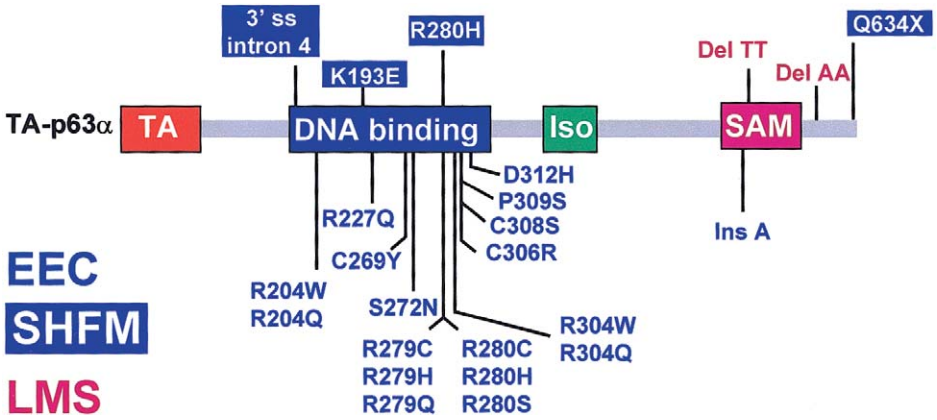

Figure 1 Distribution of *p63* gene mutations in EEC syndrome, SHFM, and LMS. Except for one frameshift mutation in exon 13, all mutations in EEC syndrome are amino acid substitutions in the DNA-binding domain that are predicted to abrogate interaction with the DNA and, hence, reduce transactivation activity. LMS mutations are frameshifts in exons 13 and 14. The EEC syndrome patient with the exon 13 frameshift mutation also has clinical characteristics of LMS. The mutations identified in patients with SHFM are distributed along the gene. Substitution of arginine 280 may give rise to either classical EEC syndrome or SHFM.

in 10 families, of which 6 were R204W and 4 were R204Q. Mutations of codon 279 occurred in eight families, R279H in four families, R279C in three families, and R279Q in one family. The R227Q mutation was detected in three unrelated families, including two families that have been reported previously (O'Quinn et al. 1998). Another frequent site of mutations is amino acid 304, which had mutation R304Q in five families and mutation R304W in two. Three families had R280C. Among families affected with EEC, single families had S272N, C306R, C308S, P309S, and D312H. Frameshift mutations in exons 13 and 14 were present in three families. An insertion of a single nucleotide (1572insA) in exon 13 was present in a patient EEC with reported elsewhere (Celli et al. 1999). A 2-bp deletion (1693–1694DelTT) in exon 13 was present in one family with LMS, and a 2-bp deletion (1860–1861DelAA) in exon 14 was present in another family with LMS. No mutation was detected in the original family with LMS (van Bokhoven et al. 1999) after sequencing of the entire coding region of the *p63* gene. Mutations were detected in 4 of 35 families with SHFM. *p63* mutations in SHFM included a missense mutation (K193E) in a father and his son, a de novo nonsense mutation (Q634X), and a de novo mutation in the 3′ splice site for exon 5. The fourth SHFM mutation (R280H) in this series was identical to a mutation observed in a patient with classical EEC syndrome, suggesting partial overlap between the EEC and SHFM mutational spectra. This patient with SHFM is a sporadic case, whose parents were unavailable for genetic testing. Thus, three of the four SHFM mutations were clearly different from those found in EEC syndrome (fig. 1).

3′ Splice-Site Mutation in Intron 4 Gives Rise to In-Frame Alternative-Splicing Products

The IVS4-2A→C mutation in a patient with SHFM abrogates the strictly conserved adenosine of the acceptor splice site. No patient material was available for direct examination, by RT-PCR, of the effect of this mutation. As an alternative strategy, an exon-trapping experiment was performed to investigate the effect of this mutation on splicing. Transfection of COS-1 cells with an exon-trapping construct containing the wild-type sequences of exon 5 and flanking intron sequences yielded transcripts that were properly spliced at the reported splice-site junctions (fig. 2). In contrast, the same construct containing the IVS4-2A→C mutation yielded transcripts that were the result of splicing at position −3 of the normal boundary between intron 4 and exon 5 (fig. 2). This result indicates that the IVS4-2A→C mutation gives rise to an insertion of a CCG codon for a proline residue at amino acid position 154.

Lack of EEC-Like Features in a Patient with a Heterozygous Deletion of the p63 *Gene*

Metaphase chromosomes from a patient carrying a deletion 3q27-3qter were used for FISH analysis to determine whether the *p63* gene was contained in the deletion. This patient had multiple congenital anomalies, including bilateral anophthalmia, congenital heart disease, and abnormal genitalia, but no features reminiscent of EEC syndrome (Chitayat et al. 1996). DNA from a YAC clone 889A7 containing the *p63* gene was labeled and was used for the FISH analysis. All cells that could be scored showed a fluorescent signal only on the normal

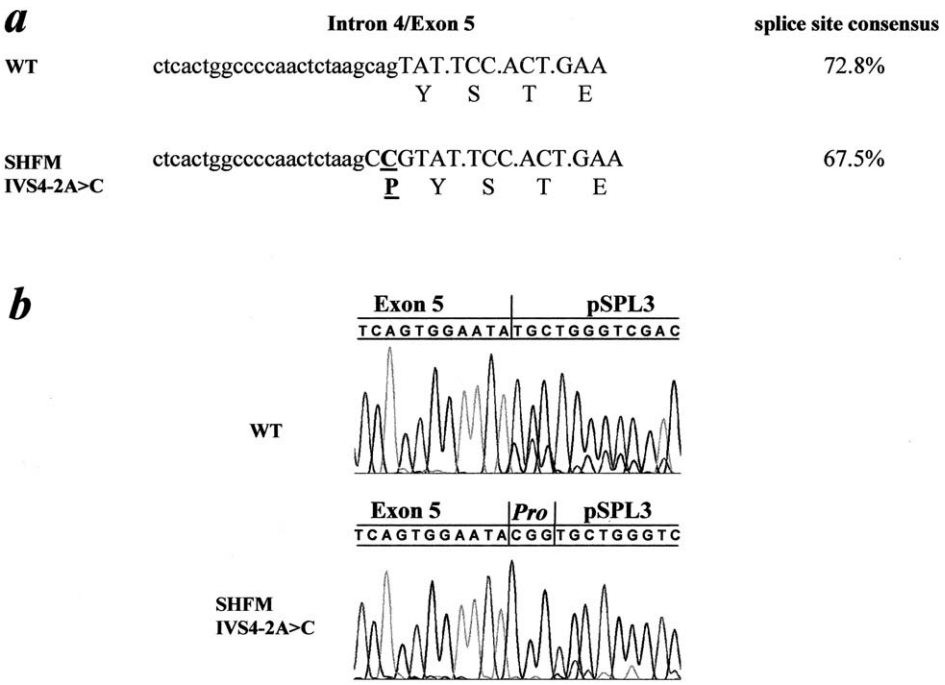

Figure 2 Predicted effect of the splice-site mutation in the *p63* gene. *a*, Predicted consequence of the 3′ splice-site mutation. The strictly conserved adenosine of the acceptor splice site has been substituted by a cytosine. An excellent alternative 3′ splice site is located just three nucleotides in front of the original one. Splicing at this alternative site results in an insertion of one proline residue for the mutant allele. The adherence to the 3′ splice-site consensus sequence was calculated according to the method of Shapiro and Senapathy (1987). *b*, The IVS4-2A→C mutation gives rise to alternative splicing. Sequence analysis of the RT-PCR product obtained in an exon-trapping assay in which COS-1 cells were transfected with either a wild-type construct (*top panel*) or a construct containing the splice-site mutation (*bottom panel*). The reverse complement sequence is shown. The mutation results in incorporation of an additional proline codon in the transcript.

chromosome 3 and not on the deletion chromosome 3 (fig. 3).

Discussion

This study shows that 40 of 43 patients with EEC syndrome have heterozygous missense mutations of the *p63* gene (table 2). From our studies a striking pattern is emerging in which mutations affecting amino acid residues 204, 227, 279, 280, and 304 are responsible for the large majority of all *p63* gene mutations in EEC syndrome (table 2). Several explanations are possible for this highly specific distribution of *p63* gene mutations.

First, the mutations may occur at nucleotides which are highly mutable. Support for this is the high frequency (≥60%) of de novo mutations in EEC syndrome (table 3). This possibility is further supported by the fact that EEC mutation hotspots in the *p63* gene correspond exactly with the sites of frequently mutated amino acids in the homologous *p53* gene in various cancers (Celli et al. 1999; Hernandez-Boussard et al. 1999). Conversely, of the five most frequently mutated

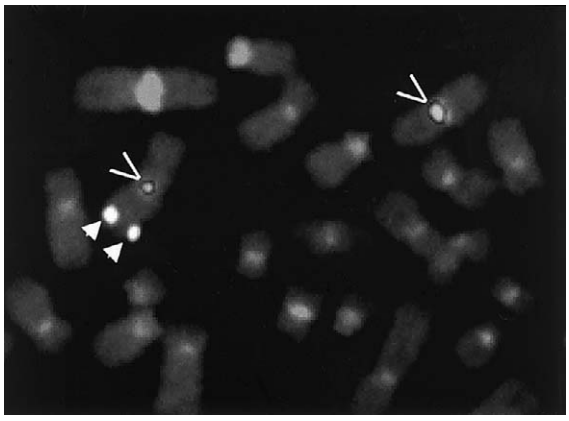

Figure 3 Partial karyotype of a patient with a terminal deletion of the long arm of chromosome 3 [46,XX,del(3)(q27→qter)]. The patient has multiple congenital abnormalities, but none of the symptoms is typical of EEC syndrome (Chitayat et al. 1996). Shown is the staining of a typical cell after FISH. Only the nondeleted chromosome 3 shows staining of the YAC containing the *p63* gene (*closed arrow*), whereas both chromosomes 3 show centromeric staining (*open arrow*).

Table 3

***p63* Mutations in Patients with EEC Syndrome, LMS, and SHFM**

Amino Acid	No. of Families	Exon	CpG Islet	Transition	De Novo Mutations[a]	Disorder
IVS4-2A→C	1	5[b]	−	A→C	0	SHFM
K193E	1	5	−	A→G	−	SHFM
R204W	6	6	+	C→T	5 +, 1 −	EEC[c]
R204Q	4	6	+	G→A	2 +, 2 −	EEC[c]
R227Q	3	6	+	G→A	−	EEC
C269Y	1	7	−	G→A	?	EEC
S272N	1	7	−	G→A	−	EEC[c]
R279H	4	7	+	G→A	3 +, 1 −	EEC
R279C	3	7	+	C→T	2 +, 1 ?	EEC
R279Q	1	7	+	GC→AA	+	EEC
R280C	3	7	+	C→T	1 +, 2 −	EEC[d]
R280S	1	7	+	C→A	?	EEC
R280H	2	7	+	G→A	1−, 1 ?	EEC/SHFM
R304W	2	8	+	C→T	1 +, 1 ?	EEC[c]
R304Q	5	8	+	G→A	4 +, 1 ?	EEC
C306R	1	8	−	T→C	+	EEC[c]
C308S	1	8	−	T→A	+	EEC
P309S	1	8	−	C→T	+	EEC
D312H	1	8	−	G→C	+	EEC
1689InsA	1	13	−	−	+	EEC[c]
1693-1694DelTT	1	13	−	−	?	LMS
1860-1861DelAA	1	14	−	−	+	LMS
Q634X	1	14	−	C→T	+	SHFM
Overall	46		34	32/34[e] C:G to T:A	27 +, 10 −, 7 ?	

[a] The question mark (?) indicates that there was insufficient clinical and/or molecular data for the parents. A plus sign (+) indicates a confirmed de novo mutation, and a minus sign (−) indicates a familial mutation.

[b] Intron mutation detected on analysis of exon 5.

[c] This mutation has been reported elsewhere by Celli et al. (1999).

[d] This mutation has also been reported in a family with SHFM (Ianakiev et al. 2000).

[e] Indicated is the number of C:G to T:A transitions at CpG sites. At non-CpG sites, 4 of 12 mutations are C:G to T:A transitions.

amino acids of *p53* in human tumor material, only one does not have a homologous mutation of *p63* in EEC syndrome. Strikingly, 34 of the 46 mutations in this series involve CpG sites. All of these 34 CpG mutations are at frequently mutated sites, whereas each of the non-CpG mutations is unique in our series (table 3). Another significant finding is that 32 of 34 CpG site mutations are C:G to T:A transitions—19 on the transcribed strand and 13 on the nontranscribed strand. The high mutability of 5-methylcytosine at CpG sites (Coulondre et al. 1978; Brown and Jiricny 1987; Rideout et al. 1990) is a likely explanation for the high proportion of the recurrent mutations in EEC syndrome. Somatic *p53* mutations in human cancers are also frequently C:G to T:A transitions at CpG dinucleotides (Hernandez-Boussard et al. 1999), and there is experimental evidence suggesting that these sites are foci for DNA damage (Denissenko et al. 1996), as well as foci for damage that is poorly repaired (Tornaletti and Pfeifer 1994). A similar mechanism may apply to the *p63* mutation hotspots identified in EEC syndrome.

A second possibility is that the mutation spectrum in EEC syndrome reflects a specific pathogenetic mechanism. This possibility is supported by the finding that a number of different missense mutations occurred at amino acids 204, 279, 280, and 304. There are several precedents in the literature for this type of selectivity. Most notably, almost all *FGFR3* gene mutations in achondroplasia occur at two amino acids, G380R and G375C (Bellus et al. 1995). This bias for specific mutations reflects a gain of function-mechanism (Li et al. 1997). Gain of function properties have previously been proposed for several missense mutations in the *p53* gene, in codons that are analogous to those mutated in EEC syndrome (Dittmer et al. 1993; Gualberto et al. 1998).

Third, the phenotypes of patients with other mutations of the *p63* gene may differ from those found in classical EEC syndrome. Such cases would not be examined in our study, which used restrictive diagnostic criteria. A number of syndromes share similarity with EEC syndrome (Rodini and Richieri-Costa 1990), notably ankyloblepharon-ectodermal defects–cleft lip/palate (AEC) syndrome (Hay and Wells 1976), acro-

dermato-ungual-lacrimal-tooth (ADULT) syndrome (Propping and Zerres 1993), and lacrimo-auriculo-dento-digital (LADD) syndrome (Hollister et al. 1973). In fact, three of four mutations found in SHFM were clearly different from those found in the group affected with EEC syndrome. Moreover, we have recently shown that missense mutations in the SAM domain (exon 13) of the *p63* gene are specifically associated with AEC syndrome (McGrath et al. 2001).

It should be noted that the three explanations offered here are not mutually exclusive. It is quite possible that EEC syndrome is, in fact, caused by specific amino acid changes that result in a change of function and also preferentially involve certain nucleotides with high mutability.

At the very least, a haploinsufficiency model can be discarded, since patients with constitutive deletions of 3q27 have none of the defining features of EEC syndrome (fig 3.; Chitayat et al. 1996). Also, mice carrying a heterozygous deletion of the *p63* gene do not have any apparent defects (Mills et al. 1999; Yang et al. 1999). In vitro expression studies of wild-type and mutant *p63* gene constructs demonstrate complex effects on transactivation and repressor functions of the six different *p63* isotypes (Celli et al. 1999; McGrath et al. 2001). Such studies also argue in favor of a dominant negative effect, a change of function, or even a partial gain of function of *p63* in EEC syndrome.

The three frameshift mutations were all in exons 13 and 14. One of these was a single-nucleotide insertion (1572insA), which we reported previously (Celli et al. 1999). This concerns a single patient with a phenotype most consistent with a diagnosis of EEC syndrome, although other features suggested LMS. The patient has bilateral typical SHFM, hypodontia, unilateral cleft lip and palate, lacrimal duct abnormalities, and dystrophic nails. In contrast with most typical EEC patients, she has normal skin. Another pertinent finding in this patient is bilateral aplasia of the mammary glands, which is more typical of LMS (van Bokhoven et al. 1999). The other frameshift mutations both occurred in patients with a phenotype that is more characteristic of LMS. The first typical LMS case has a 2-bp deletion (1576–1577delTT) in exon 13. This patient has bilateral SHFM, isolated cleft palate, and normal hair, skin, and teeth, but absent nipples. The other LMS patient has a 2-bp deletion in exon 14. This patient also has a typical LMS phenotype with bilateral SHFM, absence of the lacrimal punctae, submucous cleft palate, bilateral ear pits, and somewhat dry skin on the trunk. The nipples are absent. The anus was anteriorly placed. All three frameshift mutations affect only the p63α isotypes, which have dominant-negative effects on transactivation, whereas the β and γ isotypes may be normally produced. The two frameshift mutations in exon 13 are predicted to yield similar protein products with premature truncations in the SAM domain, whereas the frameshift in exon 14 does not affect the SAM domain (figs. 1). The combined clinical data on the three frameshift mutations suggest that they represent a specific group characterized by absence of the nipples and mammary glands and relatively mild ectodermal involvement. Such findings are unusual in EEC but are very typical in LMS (van Bokhoven et al. 1999). One of these patients, however, has been classified as having EEC syndrome, because the patient has CLP rather than CP. No mutation was detected on sequencing of the entire coding region (exons 1–15) of the *p63* gene in the large Dutch family with LMS (van Bokhoven et al. 1999). There is very clear evidence for linkage of LMS with the *p63* gene region on 3q27, with a maximum LOD score of >12 (van Bokhoven et al. 1999). It would seem likely that, in this family, LMS results from a mutation that is outside the *p63* coding region, either in an intron or in a regulatory region of the gene. Interestingly, of the three patients without a mutation who fulfilled our EEC criteria, two had cleft palate without cleft lip, and both of these had mammary gland hypoplasia. Except for the involvement of skin defects, these two cases are typical for LMS.

In this series, *p63* gene mutations were detected in only a small proportion (4/35) of patients and families with isolated SHFM. We found a novel missense mutation (K193E) in a patient with SHFM. The father of this patient has the same mutation but shows only minor foot anomalies: bilateral syndactyly I-II, bilateral small cleft II-III, and left cutaneous syndactyly III-IV (Witters et al., in press). This mutation has not been reported elsewhere, in either EEC or SHFM. However, a mutation of the adjoining amino acid (K194E) has been reported elsewhere, also in a patient with SHFM (Ianakiev et al. 2000). The K194E mutation also gives rise to variable effects, and even several cases of nonpenetrance were reported (Ianakiev et al. 2000). Two other mutations in SHFM patients are different from those found in EEC. The only nonsense mutation in this series (Q634X) occurred in a patient with SHFM, as did a mutation in the 3′ splice site in intron 4. This splice-site mutation is likely to give rise to alternative splicing rather than to skipping of exon 5, because the latter is likely to be a loss-of-function event. Three nucleotides in front of the normal 3′ splice site is a potential splice-acceptor site (fig. 2). Use of this alternative site would lead to insertion of a proline amino acid at position 233 in the DNA-binding domain. Indeed, this alternative splicing route was used for this mutation in an exon-trapping assay (fig. 2). Although unlikely, we cannot formally exclude the possibility that this mutation generates a haploinsufficiency in the patient. The fourth SHFM mutation (R280H) in our study was also

identified in a classical case of EEC syndrome. The mother of this patient with SHFM is a nonmanifesting carrier of the R280H mutation. A mutation at the same codon, R280C, has been described elsewhere in a single large South African family with SHFM (Ianakiev et al. 2000). Conversely, we detected the R280C mutation in three unrelated cases with classical EEC syndrome, including nail dysplasia, dry skin, orofacial clefting, and hypodontia. At least seven members of the South African family with SHFM received an in-depth clinical investigation, and no evidence for ectodermal involvement or clefting was found (Ianakiev et al. 2000). These data strongly suggest that codon 280 mutations can cause either EEC or SHFM. A similar situation has previously been described for the *FGFR2* gene, in which some mutations are specifically associated with either Crouzon syndrome or Pfeiffer syndrome, but others, like the Cys342Arg mutation, were found in both these conditions or in Jackson-Weiss syndrome (Rutland et al. 1995; Tartaglia et al. 1997).

In conclusion, we show that most, if not all, patients with EEC syndrome have *p63* gene mutations. Genotype-phenotype correlations exist in that the specific mutations in EEC syndrome are not generally found in SHFM or LMS. With the exception of a frameshift mutation in exon 13, these mutations change specific amino acids in the DNA-binding domain (exons 5–8 of the gene). LMS patients are likely to have frameshift mutations near the 3′ end of the gene. The mutation spectrum for SHFM is different and includes nonsense and splice-site mutations, as well as missense mutations not usually found in EEC. The proportion of SHFM patients with *p63* gene mutations is low, and the majority of SHFM cases are likely to be due to mutations in other genes. The high phenotypic variability that is observed for specific mutations—in particular, the finding that mutations at codon 280 can cause either EEC or SHFM in different families—suggests the importance of modifying factors, the nature of which remains to be determined.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/index.html (for *p63* gene sequences [accession numbers AF075430 and AF091627])

IARC *p53* Mutation Database, http://www.iarc.fr/p53/index.html (for the distrubution and frequencies of mutations in the *p53* gene)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for SHFM [MIM 183600, 600095, and 313350], and EEC [MIM 129900 and 602077])

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