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Spotlight on p63

p63-Associated Disorders

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ABSTRACT

Heterozygous mutations in the transcription factor gene ***p63*** are causative for several syndromes with ectodermal dysplasia, orofacial clefting and limb malformations as the key characteristics. Different combinations of these features are seen in five different syndromes, of which ectrodactyly, ectodermal dysplasia and cleft lip/palate syndrome (EEC) is the most common one. Mutations in ***p63*** can also cause non-syndromic single malformations, such as split hand foot malformation (SHFM4) and isolated cleft lip (NSCL). In this article we will present an overview of diseases caused by mutations in the ***p63*** gene and review the known pathogenic ***p63*** gene mutations.

INTRODUCTION

The transcription factor *p63* is a key regulator of ectodermal, orofacial and limb development. This became apparent in 1999, by the generation of *p63* knockout mice[1,2] and by the finding of dominant mutations in human disorders with ectodermal dysplasia, split hand/foot malformation and orofacial clefting.[3-7] Mutations in the *p63* gene can cause at least five different syndromes: ectrodactyly, ectodermal dysplasia and cleft lip/palate syndrome (EEC, OMIM 604292), ankyloblepharon-ectodermal defects-cleft lip/palate syndrome (AEC, OMIM 106260), limb lammary syndrome (LMS, OMIM 603543), acro-dermato-ungual-lacrimal-tooth syndrome (ADULT, OMIM 103285) and Rapp-Hodgkin syndrome (RHS, OMIM 129400). Furthermore, two non-syndromic human disorders are caused by *p63* mutations: isolated split hand/foot malformation (SHFM4, OMIM 605289) and recently non-syndromic cleft lip.[8] Here we present an overview of these seven *p63*-linked conditions, the genotype-phenotype associations and give an update of all known pathogenic *p63* mutations.

p63 PHENOTYPE

Ectodermal dysplasia (ED) is one of the three main characteristics of the *p63*-associated syndromes (Fig. 1). Ectodermal dysplasia manifests as the abnormal development or growth of tissues and structures that are developed from the outer embryonal layer, ectoderm. In this condition skin, hair, teeth, nails and several exocrine glands, such as sweat and sebaceous glands are usually abnormally developed. The epidermis of *p63* patients can be very dry, itchy and hypopigmented. In extreme cases widespread areas of the skin can be erosed often including the scalp. This is most common in the AEC syndrome, where patients present patches of life-threatening congenital skin erosion. However, the skin usually recovers after the first year. The amount of scalp and body hair is often diminished and hair can be wiry or curly. Alopecia is sometimes reported. Occasionally the eyelashes and eyebrows are also absent. The number of teeth is often less than in healthy individuals, indicating that there is a reduced number of teeth placodes. Teeth can also be malformed by a conical shape and poor enamel formation, causing subsequent tooth decay. Nails can be dystrophic, thickened and discoloured. The absence or reduced amount of sweat glands is also reported among *p63* patients and leads to diminished perspiration, which can be life-threatening. Mammary gland and nipple hypoplasia are other manifestations of the ectodermal dysplasia spectrum observed in *p63* patients. Also the development and function of sebaceous and salivary glands are frequently abnormal. Lacrimal duct defects and obstruction of the lacrimal ducts impose a risk for conjunctivitis and corneal damage. Characteristics of ED described here varies between the *p63* syndromes and even within a one single syndrome, as will be discussed below in this article.

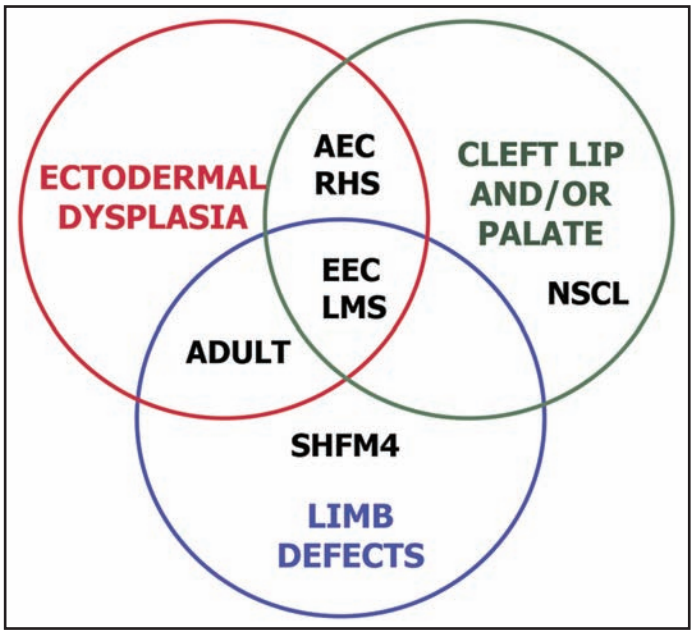

Figure 1. Various combinations of ectodermal dysplasia, orofacial clefting and limb malformations are the hallmark of p63-associated syndromes. EEC syndrome is the prototype of these syndromes and together with LMS shows all three hallmarks. ADULT syndrome patients never show orofacial clefting, whereas AEC and RHS never show limb defects. Non-syndromic limb defect condition (SHFM4) and non-syndromic cleft lip/palate (NSCL) are also caused by mutations in the p63 gene.

Split hand/foot malformation (SHFM) constitutes the second part of the *p63* syndrome phenotype (Fig. 1). Hands and feet are often malformed and have a severe median cleft in the palm and/or in the sole. These clefts usually occur in conjunction with a lack of one or more central (2-3-4) digits, which is called ectrodactyly. Fusion of fingers or toes, which is called syndactyly, can be seen in conjunction with ectrodactyly. In most cases SHFM is part of a syndrome, but it can also be present in isolation, without other symptoms.

The third hallmark of *p63* syndrome phenotype is orofacial clefting (Fig. 1). It is mainly seen in the form of cleft lip (CL) and/or cleft palate (CP). This symptom is usually observed as part of a complex syndrome, where other organs are also affected, however, recently Leoyklang et al.[8] have described mutations in *p63* gene causing orofacial clefting as the only abnormality.

Most of the above developmental defects are also observed in *p63* knockout mice, which have particular severe defects of ectodermal structures and the limbs.[1,2] Heterozygous $p63^{+/-}$ mouse have no significant developmental abnormalities, suggesting that loss-of-function of one allele is not disease-causing. Recently, it has been reported that a high proportion of $p63^{+/-}$ aging mice develop tumours indicating a role of *p63* in tumour suppression.[9] However, this observation could not be confirmed in an independent study.[10] There is no indication that individuals with heterozygous *p63* mutation are prone to develop tumours.

ALLELIC *p63* CONDITIONS

EEC syndrome. The prototype of the *p63* syndrome family is the EEC syndrome (Fig. 1). EEC syndrome patients are invariably characterized by one or more features of ectodermal dysplasia, which can present as defects of hair, skin, nails, teeth and glands. The severity and type of the ectodermal features is highly variable, and to some extent dependent on the exact nature of the mutation (see below). Only few patients show defects in all of the described ectodermal structures above. EEC patients occasionally also have mammary gland/nipple hypoplasia (14%) and hypohidrosis (11%). About two-thirds of these patients have ectrodactyly, and syndactyly is also frequent (43%). Cleft lip/palate is present in about 40% of the EEC patients, mostly as CL with or without CP.[11]

EEC syndrome is mainly caused by point mutations in the DNA binding domain (DBD) of the *p63* gene (Table 1). Altogether 34 different mutations have been reported, and 20 different amino acids are involved. Only two mutations are outside the DNA binding domain: one insertion (1572 InsA) and one point mutation (L563P) in the sterile α motif domain (SAM).[4,11] In earlier studies we found five frequently mutated amino acids: R204, R227, R279, R280 and R304 in the EEC population, all located in CpG islands (Fig. 2). These five mutations explain almost 90% of the EEC syndrome patients.[11,12] The five p63 arginine hotspot mutations and probably also other DNA binding domain mutations that are found in EEC syndrome appear to impair the p63 protein binding to DNA.[4] The autosomal dominant inheritance of EEC syndrome suggests that the EEC mutations have a dominant negative effect. However, recent genotype-phenotype analyses for the five hotspot mutations revealed significant differences between the corresponding phenotypes. For instance cleft lip/palate is present in the R304 mutation population (80%), whereas R227 patients seldom have cleft lip/palate. Syndactyly is completely absent in R227 population, while 30-60% of the other hotspot mutation population have syndactyly. Genitourinary defects are frequently observed in R227 mutation population (40%), while significantly less in other populations.[11] It thus seems that these hotspot mutations exert specific effects. Such specificity might be brought about by different effects of these mutations on promoters for p63 transcriptional target genes. Alternatively, these hotspot mutations may exhibit gain-of-function effects, similar as for the p53 hotspot mutations.[13]

Limb mammary syndrome (LMS). The LMS phenotype resembles the EEC syndrome phenotype, but the ectodermal manifestations are milder (Fig. 1).[3] A consistent feature of LMS is the mammary gland and/or nipple hypoplasia (100%). Lacrimal duct obstruction and dystrophic nails are frequently observed (59 and 46% respectively), hypohydrosis and teeth defects are detected in about 30%, but other ectodermal defects such as hair and skin defects are rarely detected if at all. About 70% of LMS patients have similar limb malformations as in EEC syndrome, and about 30% orofacial clefting, notably always in form of cleft palate.[11]

Mutations in LMS are located in the N- and C-terminus of the *p63* gene (Table 1). A large LMS family (29 affected members) has a point mutation in exon 4, causing a G76W substitution in the ΔN-specific putative transactivation domain (TA2).[3,12,14,15] One other point mutation (S90W) is also located between the TA domain and DBD. Other LMS mutations are reported in the C-terminus: a TT deletion in the exon 13 and an AA deletion in exon 14.[6,12] These will affect only the p63α protein isoforms, where they are predicted to cause a frame shift and a premature stop codon. Also a stop mutation in the transcription factor inhibitory domain (TI) (K632X) has been identified in a sporadic LMS patient.[11] The latter mutation is predicted to impair the suppressive effect of the TI domain towards the TA domain, thus increasing the transactivation activity.[16]

ADULT syndrome. ADULT syndrome phenotype is most similar to LMS syndrome, although clear differences can be seen when

Table 1 **Pathogenic *p63* mutations in seven allelic diseases**

Mutation	Exon	Domain	Isoform		Syndrome	Reference
N6H	3'	TA2	dN	alpha, beta, gamma	ADULT	Amiel et al. 2001
R58C	3	TA	TA	alpha, beta, gamma	SHFM	Zenteno et al. 2005
G76W	4	-	dN, TA	alpha, beta, gamma	LMS	van Bokhoven and Brunner 2002
S90W	4	-	dN, TA	alpha, beta, gamma	LMS	Authors' unpublished data
3'ss intron 4	intron 4/ exon 5	-	dN, TA	alpha, beta, gamma	SHFM	van Bokhoven et al. 2001
G134D	4	-	dN, TA	alpha, beta, gamma	LMS	Authors' unpublished data
G134D	4	-	dN, TA	alpha, beta, gamma	ADULT	Slavotinek et al. 2005
L162P	5	DBD	dN, TA	alpha, beta, gamma	EEC	Rinne et al. 2006
Y163C	5	DBD	dN, TA	alpha, beta, gamma	EEC	van Bokhoven and Brunner 2002
Y192C/D	5	DBD	dN, TA	alpha, beta, gamma	EEC	Authors' unpublished data
K193E	5	DBD	dN, TA	alpha, beta, gamma	SHFM	van Bokhoven et al. 2001
K194E	5	DBD	dN, TA	alpha, beta, gamma	SHFM	Ianakiev et al. 2000
V202M	5	DBD	dN, TA	alpha, beta, gamma	EEC	van Bokhoven and Brunner 2002, Pozo et al 2004
R204L/Q/W	6	DBD	dN, TA	alpha, beta, gamma	EEC	Celli et al. 1999, van Bokhoven et al. 2001, de Mollerat et al. 2003, Berdon Zapata et al. 2004, van Bokhoven en Brunner 2002, Rinne et al. 2006
H208Y	6	DBD	dN, TA	alpha, beta, gamma	EEC	Rinne et al. 2006
R227Q	6	DBD	dN, TA	alpha, beta, gamma	EEC	van Bokhoven and Brunner 2002, Rinne et al. 2006
C269Y	7	DBD	dN, TA	alpha, beta, gamma	EEC	van Bokhoven et al. 2001
S272N	7	DBD	dN, TA	alpha, beta, gamma	EEC	Celli et al. 1999
C273Y	7	DBD	dN, TA	alpha, beta, gamma	EEC	van Bokhoven et al. 2001
R279C/H/Q	7	DBD	dN, TA	alpha, beta, gamma	EEC	Celli et al. 1999, Ianakiev et al. 2000, Kosaki et al. 2001, van Bokhoven and Brunner 2002, South et al. 2002, Dianzani et al. 2003, de Mollerat et al. 2003, Berdon-Zapata et al. 2004
R279H	7	DBD	dN, TA	alpha, beta, gamma	RHS	Bougeard et al. 2003
R280C/H/S	7	DBD	dN, TA	alpha, beta, gamma	EEC	van Bokhoven et al. 2001, van Bokhoven and Brunner 2002, Barrow et al. 2002, Ray et al. 2004
R280C/H	7	DBD	dN, TA	alpha, beta, gamma	SHFM	Ianakiev et al. 2000
R298G/Q	8	DBD	dN, TA	alpha, beta, gamma	ADULT	Propping et al. 2000, Duijf et al. 2002, Chan et al. 2004, Rinne et al. 2006
R304P/Q/W	8	DBD	dN, TA	alpha, beta, gamma	EEC	Celli et al. 1999, Ianakiev et al. 2000, Wessagowit et al. 2000, van Bokhoven et al. 2001, Hamada et al. 2002, Dianzani et al. 2003, de Mollerat et al. 2003
C306Y/R	8	DBD	dN, TA	alpha, beta, gamma	EEC	Celli et al. 1999, Lehmann et al. 2005
C308S/Y	8	DBD	dN, TA	alpha, beta, gamma	EEC	van Bokhoven et al. 2001
P309S	8	DBD	dN, TA	alpha, beta, gamma	EEC	van Bokhoven et al. 2001
D312G/H/N	8	DBD	dN, TA	alpha, beta, gamma	EEC	van Bokhoven et al. 2001, Akahoshi et al. 2003
R313G	8	DBD	dN, TA	alpha, beta, gamma	EEC	Authors' unpublished data
R313G	8	DBD	dN, TA	alpha, beta, gamma	NSCL	Leoyklang et al. 2006
A315E	8	DBD	dN, TA	alpha, beta, gamma	EEC	Authors' unpublished data
3'ss intron 10	intron 10/ exon 11	-	dN, TA	alpha, beta, gamma	AEC	Barrow et al. 2002
I510T	13	SAM	dN, TA	alpha	RHS	Bertola et al. 2004
I510T	13	SAM	dN, TA	alpha	AEC	Bertola et al. 2004, Sorasio et al. 2006
L514F/S/V	13	SAM	dN, TA	alpha	AEC	McGrath et al. 2001, Payne et al. 2005
G518V	13	SAM	dN, TA	alpha	AEC	Authors' unpublished data
C522G/W	13	SAM	dN, TA	alpha	AEC	McGrath et al. 2001
1572 Ins A	13	SAM	dN, TA	alpha	EEC	Celli et al. 1999
1576 Del TT	13	SAM	dN, TA	alpha	LMS	van Bokhoven and Brunner 2002
G530V	13	SAM	dN, TA	alpha	AEC	McGrath et al. 2001
T533P	13	SAM	dN, TA	alpha	AEC	McGrath et al. 2001
534 Ins TTC	13	SAM	dN, TA	alpha	AEC	Tsutsui et al 2003
Q536L	13	SAM	dN, TA	alpha	AEC	McGrath et al. 2001
I537T	13	SAM	dN, TA	alpha	AEC	McGrath et al. 2001, van Bokhoven and Brunner 2002
S541F	13	SAM	dN, TA	alpha	AEC	Authors' unpublished data
S541P/Y	13	SAM	dN, TA	alpha	RHS	Kantaputra et al. 2003, Shotelersuk et al. 2005
R555P	14	SAM	dN, TA	alpha, beta	AEC	Payne et al. 2005
I558T	14	SAM	dN, TA	alpha, beta	AEC	Authors' unpublished data
L563P	14	SAM	dN, TA	alpha, beta	EEC	Rinne et al. 2006
1709 Del A	14	TI	dN, TA	alpha, beta	RHS	Bougeard et al. 2003
1721 Del C	14	TI	dN, TA	alpha, beta	RHS	Kannu et al. 2006, Rinne et al. 2006
1742 Del C	14	TI	dN, TA	alpha, beta	AEC	van Bokhoven and Brunner 2002
1743 Del AA	14	TI	dN, TA	alpha, beta	LMS	van Bokhoven et al. 2001
1787 Del G	14	TI	dN, TA	alpha, beta	RHS	Chan et al. 2005
1859 Del A	14	TI	dN, TA	alpha, beta	RHS	Dianzani et al. 2003
1859 Del A	14	TI	dN, TA	alpha, beta	AEC	Authors' unpublished data
K632X	14	TI	dN, TA	alpha, beta	LMS	Rinne et al. 2006
Q634X	14	TI	dN, TA	alpha, beta	SHFM	van Bokhoven et al. 2001
E639X	14	TI	dN, TA	alpha, beta	SHFM	van Bokhoven and Brunner 2002

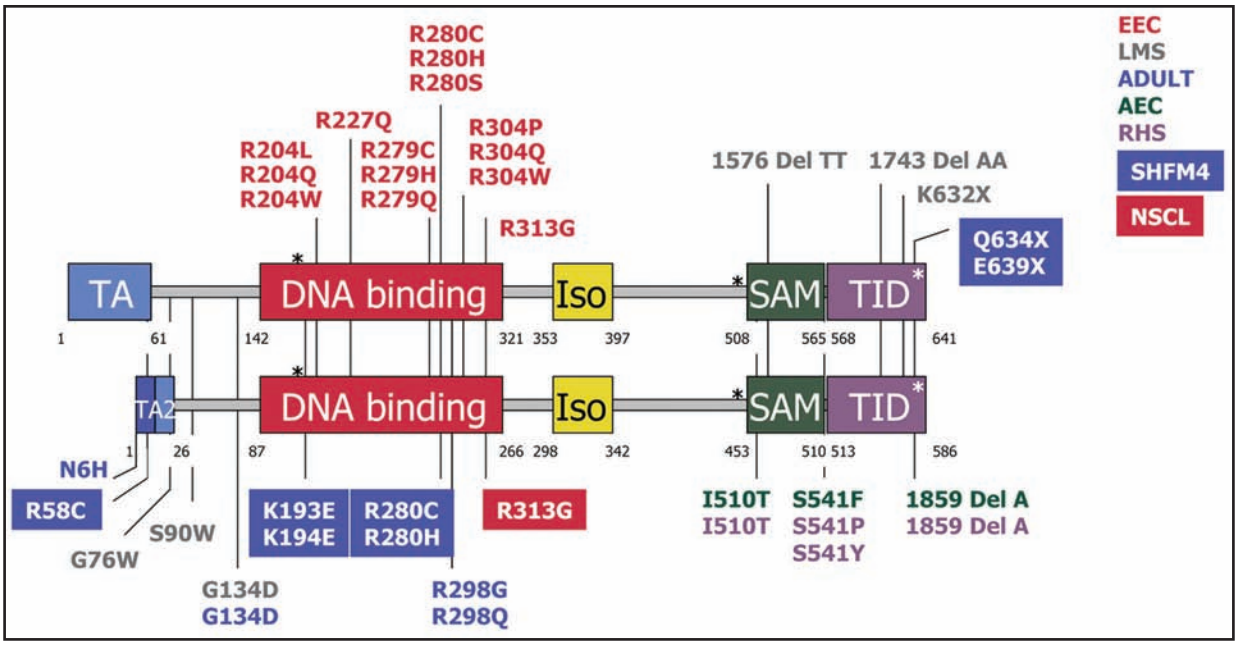

Figure 2. Pathogenic *p63* mutations cause at least five different syndromes and two non-syndromic conditions. Mutations causing different diseases are illustrated in different colours. Only mutations that are discussed in the text are indicated, an overview of all mutations is given in Table 1. EEC hotspot mutations are clustered in DNA binding domain, and RHS and AEC syndrome mutations in SAM and TI domains. Several mutations, such as R280, R313, I510, S541 and 1850 Del A, can have a variable clinical outcome, probably due to genetic background effects. The black asterisks illustrate sites needed for upiquitination (K193, K194 and PY) and the white asterisk represents a sumoylation site (fKXD/E).[40,42,58]

observing larger families or patient populations. The main difference is the absence of orofacial clefting and the presence of hair and skin defects in the ADULT syndrome (Fig. 1). Teeth, skin and nail defects are constantly present in ADULT syndrome (100, 93 and 100%, respectively), but only rarely in LMS. Hair (53%) and lacrimal duct defects (67%) are observed in ADULT patients more frequently than in LMS. Freckling has been reported, but cannot be considered to be a differentiating feature of this syndrome.[17]

By today, four ADULT syndrome families and three sporadic cases have been reported.[17-23] All the families and one of the sporadic cases have a point mutation in exon 8, changing R298 in the DNA binding domain into either a glutamine or a glycine (Table 1). While EEC syndrome mutations in the DNA binding domain impair the binding of p63 protein to DNA,[4] arginine 298 is not located close to the DNA-binding interface, and mutation of this arginine does not affect DNA binding.[21] Instead, earlier studies have shown a gain-of-function effect for the mutated ΔNp63γ isoform, which usually does not have a transactivation activity in assays using an optimized *p53*-responsive element.[17,21] Two other mutations are located in the N-terminus: N6H mutation affects only the ΔN-isoforms and in another isolated patient a missense mutation G134D* is located just front of the DBD in exon 4.[20,23]

AEC syndrome. The AEC syndrome phenotype differs from the other conditions mainly by the severity of the skin phenotype, the occurrence of an eyelid fusion at birth and the absence of limb malformations (Fig. 1). Approximately 80% of the patients have severe skin erosion at birth, which usually will recover in the first years of the life. The eyelid fusion, also called ankyloblepharon, is present in about 45% of AEC patients, but only rarely in other *p63*-associated conditions. The other ED symptoms, such as nail and teeth defects are present in more than 80% of patients, and hair defects and/or alopecia are almost constant features (94%). Lacrimal duct obstruction is seen in 50% of patients, whereas mammary gland hypoplasia and hypohydrosis occur occasionally (both 13%). Interestingly, almost 40% of patients have hearing impairment and genito-urinary defects. Cleft lip is present in 44% and cleft palate in about 80%. Limb malformations are almost absent. Ectrodactyly has never been reported, but 25% of patients has only mild syndactyly.[11]

Rapp-Hodgkin syndrome (RHS). RHS mimics AEC very much (Fig. 1). The differences discussed earlier in several papers are the absence of ankyloblepharon in RHS and the more severe skin phenotype in AEC.[11,24] Other ED symptoms, such as orofacial clefting and the near absence of limb malformations are similar to AEC. These two conditions could be considered as a single entity, since the ankyloblepharon is present only in about 45% of AEC syndrome patients, and therefore is not a discriminating factor. Although, the severity of the skin phenotype is obvious and more severe in AEC patients than in RHS patients, the strong overlap between AEC and RHS suggest, that they are variable manifestations of the same clinical entity.[11,24]

AEC and Rapp Hodgkin syndrome mutations are located in the C-terminus of the p63 protein (Fig. 2). They are either point mutations in the SAM domain or deletions in the SAM or TI domains (Table 1).[7,11,12,24-35] The SAM domain is known to be involved in protein-protein interactions and therefore mutations in this domain are most probably hampering the binding to interacting proteins. One known interactor of p63 SAM domain is the Apobec-1-binding protein-1 (ABBP1), which is a member of RNA processing machinery and known to regulate the alternative splicing of the Fibroblast-growth-factor-receptor-2 (FGFR2) towards the epithelial specific isoform. AEC mutations in the SAM domain abolish the binding to ABBP1, which most probably leads to changes in FGFR2 RNA splicing.[36] Interestingly, gain-of-function mutation

*In the article incorrect amino acid annotation, erratum pending ref. Slavotinek

in FGFR2 gene have been reported in a number of craniosynostosis syndromes, which are also characterized by distal limb malformations.[37] Recently, loss-of-function mutations in FGFR2 have been found in lacrimo-auricolo-dento-digital syndrome (LADD), an ED syndrome characterized by dominant inheritance of limb defects in association with abnormal lacrima, ear cups and teeth.[38] Interestingly, LADD shows marked overlap with EEC syndrome, and was earlier also presumed to be caused by mutations in the *p63* gene.

NON-SYNDROMIC *p63* CONDITIONS

Split hand/foot malformation type 4 (SHFM4) is a "pure" limb malformation (ectrodactyly and syndactyly) condition, thus without orofacial clefting or ectodermal dysplasia. The non-syndromic SHFM4 is caused by several mutations, which are dispersed throughout the *p63* gene: a point mutation in the transactivation domain (TA) (R58C), a splice-site mutation in front of exon 4 (3'ss intron 4), four missense mutations in the DNA binding domain (K193E, K194E, R280C, R280H), and two nonsense mutations in the TI-domain (Q634X, E639X) (Table 1).[5,6,39] It is still unclear how these widely dispersed mutations cause the limb defect. Interestingly, several SHFM4 mutations are reported to cause alteration in the p63 protein activation and stability: Q634X and E639X are known to disrupt the sumoylation site, and therefore increase the stability and transcriptional activity of the p63α isoform.[40,41] Furthermore, amino acids K193 and K194 are required for ubiquitin conjugation by E3 ubiquitin ligase (Itch) and naturally occurring mutations in those amino acids cause more stable p63 protein.[42] Possibly, SHFM is caused by altered protein degradation, even though different degradation routes are involved. Another divergent phenomenon is the R280C/H mutations, which are not only causative for SHFM4, but also for the syndromic EEC phenotype in other families. In such families, the phenotype is always consistent, either EEC or SHFM4. In SHFM4 families decreased penetrance is reported, for the R280 mutation, suggesting an effect of a nearby genetic modifier.

Recently, a non-syndromic orofacial clefting type was also linked to *p63* gene.[8] The amino acid change R313G is the first mutation causing the non-syndromic cleft lip/palate (NSCL) phenotype in the *p63* gene.[8] This mutation was also observed in a sporadic EEC syndrome phenotype (authors' unpublished data). Mutations and/or polymorphisms in 3 other genes, *IRF6*, *MSX1* and *PVRL*, are also associated with a syndromic and non-syndromic forms of orofacial clefting.[43-49] In the study of Leoyklang et al.[8] also other changes in the *p63* gene were found (N87N, S90L, L248L, H406H, D564H), but because the mutations did not change the amino acid sequence or were also found in one of the healthy parents or in the control population, they were not considered to be pathogenic changes. Nevertheless, since orofacial clefts are considered to have a multifactorial origin it is quite possible that these changes in the *p63* gene impose a risk factor for facial clefting type, similar as was reported for the *IRF6* gene.

DISCUSSION

The mutation patterns in the *p63* gene, that are associated with different *p63* clinical conditions display a clear genotype-phenotype association: especially in the EEC and AEC/RHS syndromes, where mutations are clustered in the DNA binding domain and in the SAM and TI domains, respectively. In about 250 patients we and others have described 74 different mutations causing five different syndromes and two different non-syndromic conditions. This still increasing number of patients allows the delineation of the phenotypic and genotypic pictures of different *p63*-associated disorders. In addition, evidence is accumulating that other genetic factors influence the final *p63* mutant phenotype.

Several examples show that the same mutation can lead to different clinical conditions. AEC and RHS syndromes share three mutations: I510, S541 and 1859 Del A.[24,25,28,29,31,33] Often the clinical distinction between RHS and AEC syndrome is solely based on the presence or absence of ankyloblepharon, which in fact is not the discriminating feature between these two conditions.[11] Also LMS and ADULT syndrome seem to be caused by the same mutation G134D (authors' unpublished data),[23] as well as EEC syndrome and non-syndromic cleft lip/palate due to the R313G mutation (authors' unpublished data).[8] Also several frameshift mutations are found, interestingly always with variable phenotype, thus causing EEC, LMS, AEC or RHS syndrome. The possible explanation may be a misdiagnose in sporadic cases, because of the phenotypic variability, likely, however frameshift mutations are exceptionally sensitive to the effects of modifier genes. An example of family-dependent clinical phenotype is the arginine 280 mutation, which can be mutated to cysteine or histidine and lead either to the non-syndromic SHFM4 (2 families) or more often to syndromic ectodermal dysplasia with SHFM and orofacial clefting (EEC) (9 families).[5,6,26,50] Surprisingly, the penetrance is reduced in SHFM families, but not in EEC families, which also indicates a modifier effect somewhere else in the genome.

The phenotypic variation between the *p63*-linked diseases is large, furthermore the phenotypic variation within one disease is also considerable. It is clear that variability within families may be ascribed to a combination of modifier genes, and stochastic processes. No such modifier genes have yet been identified for the *p63* syndromes described in this review. However, modifiers have been identified for the phenotypic effects of *p53* gene mutations in human cancer, and at least one of these has also been shown to affect p63 protein levels and transcription.[51,52] Other candidate modifier genes may be found in pathways that are known to affect specific phenotypes. For instance, genetic variation in the *IRF6* gene could be tested as a risk factor for cleft lip in *p63* mutant families.[47-49]

Furthermore, variation between families likely reflects differential downstream effects for each of these mutations. There are many possible ways in which this can be achieved, as the p63 protein is expressed in several isoforms, which are involved in different cellular functions during the embryonal development but also later on. The variation in the phenotypes caused by each of the five EEC hotspot mutations is also striking. The EEC hotspot mutation data, where clear phenotypic variation is seen between the mutations inside a single clinical syndrome, might reflect that different target genes are affected by each of the hotspot mutations. Whether this is so, needs to be examined in more detail in animal models or cellular systems. Clearly, these EEC mutations cannot just be simple loss-of-function or dominant negative mutations, but gain-of function mutations are likely involved, as was described for *p53* mutations in transgenic mice.[53-55]

Finally, the finding that some mutations such as R280C/H have different phenotypes between families, but the same phenotype consistently within families suggest the influence of cis-acting polymorphisms, either in *p63* itself or within a genetically linked gene on chromosome 3q. Such functional polymorphisms have not yet been described, but here too, there are precedents for the existence of such polymorphisms in both *p53* and *p73*.[56,57] Each of these

possible mechanisms for phenotypic modulation will require functional hypotheses that can be verified in experimental systems, and ultimately be tested for their impact on the many families that are affected by these clinically often severe malformations.

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