



## A novel c.1037C > G (p.Ala346Gly) mutation in *TP63* as cause of the ectrodactyly-ectodermal dysplasia and cleft lip/palate (EEC) syndrome

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### Abstract

Ectrodactyly – ectodermal dysplasia and cleft lip/palate (EEC) syndrome (OMIM 604292) is a rare disorder determined by mutations in the *TP63* gene. Most cases of EEC syndrome are associated to mutations in the DNA binding domain (DBD) region of the p63 protein. Here we report on a three-generation Brazilian family with three individuals (mother, son and grandfather) affected by EEC syndrome, determined by a novel mutation c.1037C > G (p.Ala346Gly). The disorder in this family exhibits a broad spectrum of phenotypes: two individuals were personally examined, one presenting the complete constellation of EEC syndrome manifestations and the other presenting an intermediate phenotype; the third affected, a deceased individual not examined personally and referred to by his daughter, exhibited only the split-hand/foot malformation (SHFM). Our findings contribute to elucidate the complex phenotype-genotype correlations in EEC syndrome and other related *TP63*-mutation syndromes. The possibility of the mutation c.1037C > G being related both to acro-dermato-ungual-lacrimal-tooth (ADULT) syndrome and SHFM is also raised by the findings here reported.

**Keywords:** EEC syndrome, *TP63*-mutations, p63-associated disorders, SHFM.

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Ectrodactyly-ectodermal dysplasia and cleft lip/palate (EEC) syndrome (OMIM 604292) is a rare autosomal-dominant disorder characterized by dry or eczematous skin, sparse hair on the scalp, eyebrows and eyelashes, nail dystrophy and hypodontia, in addition to ectrodactyly and orofacial cleft. Other clinical manifestations include syndactyly, absence or reduced number of sweat, sebaceous, and salivary glands, mammary gland/nipple hypoplasia, nasolacrimal duct abnormalities, conductive or sensorineural hearing loss, facial dysmorphisms, chronic infections of the respiratory tract, genitourinary anomalies, and development delay (Clements *et al.*, 2009; Celli *et al.*, 1999; Shivaprakash *et al.*, 2012). EEC is a complex pleiotropic condition of multiple congenital dysplasia and anomalies. The clinical diagnosis of the syndrome is hampered by its extremely variable expression (Rodini and Richieri-Costa, 1990; Celli *et al.*, 1999; Rinne *et al.*, 2006). The penetrance is practically complete and just a few cases of arguable lack of penetrance have been reported (Roelfsema and Cobben, 1996).

Most EEC cases, if not all, result from mutations in the *TP63* gene, and more than 200 cases have already been reported in the literature (Clements *et al.*, 2009). EEC is one out of five distinct ectodermal dysplasia (ED) syndromes caused by mutations in this gene, which include, besides EEC, acro-dermato-ungual-lacrimal-tooth syndrome (ADULT, OMIM 103285), ankyloblepharon-ectodermal defects-cleft lip/palate syndrome (AEC, OMIM 106260), limb-mammary syndrome (LMS, OMIM 603543), and Rapp-Hodgkin syndrome (RHS, OMIM 129400). Nonsyndromic split hand/foot malformation (SHFM4, OMIM 605289) and, very rarely, nonsyndromic cleft lip may be caused by mutations in this same *TP63* gene (Leoyklang *et al.*, 2006; Rinne *et al.*, 2006). All conditions related to *TP63* mutations exhibit clinical features that largely overlap with EEC syndrome, complicating the differential diagnosis among them (Clements *et al.*, 2009; Celik *et al.*, 2011).

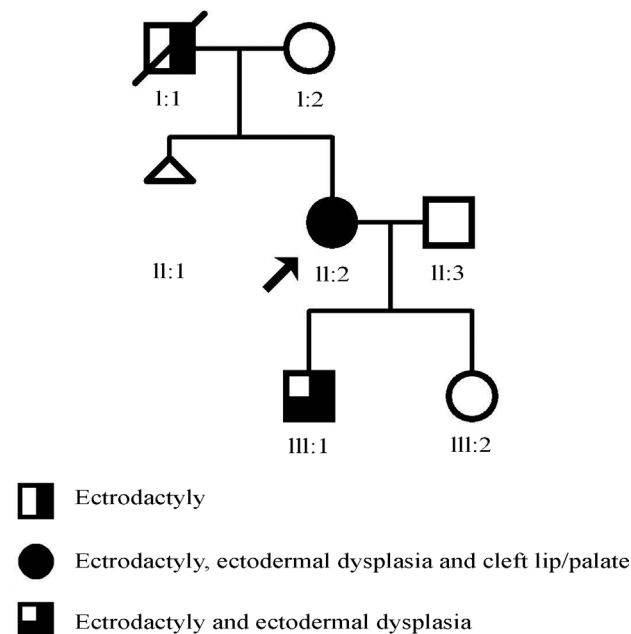
The protein p63, encoded by *TP63* gene on 3q27, is a member of p53 family. Like the *TP53* and *TP73* genes, *TP63* encodes a sequence-specific transcription factor that activates target genes involved in different cellular pathways. However, unlike the p53 protein, p63 protein is not involved in tumor suppression, but plays an important role as a transcription factor involved in limb, epithelial, and

craniofacial development (Rinne *et al.*, 2007; van Bokhoven *et al.*, 2011). p63 is expressed as at least six different isoforms, three of which include a transactivation domain (TAp63) and the other three, none ( $\Delta$ Np63). Alternative splicing produces additional three C-terminal regions,  $\alpha$ ,  $\beta$  and  $\gamma$  isoforms.  $\Delta$ Np63 $\alpha$  is the major isoform expressed in the epidermis (Browne *et al.*, 2011). The EEC syndrome is mainly caused by missense mutations in the DNA binding domain (DBD), a region with transactivation activity present in all isoforms (Celli *et al.*, 1999; Rinne *et al.*, 2007).

Here we report a novel heterozygous missense mutation in *TP63* (c.1037C > G) that results in p.Ala346Gly, causing EEC in a Brazilian family (Figure 1).

The proband, a woman born in the Northern region of Brazil, presents the complete manifestation of the EEC syndrome (Figure 2), bilateral split hand and split foot malformation (SHFM), cleft lip and palate, dracryocystitis (secondary to obstruction of the lacrimal duct) with corneal ulceration, blepharitis, blepharophimosis, bilateral sensorineural hearing loss, hypodontia (Figure 2A, C and E). In addition, she had hypothyroidism.

Her affected son also had bilateral SHFM. In the lower limbs, the splitting was accompanied by malformation of the leg bones, probably tibial and/or fibular hemimelia (lower limb extremities and distal region of the leg bones were amputated and no X-rays were available). He also presented dracryocystitis, hypodontia, thin and hypopigmented hair, blepharitis, and blepharophimosis, but no labiopalatine cleft (Figure 2B and D).



**Figure 1** - Pedigree of family with ectrodactyly-ectodermal dysplasia and cleft lip/palate (EEC) syndrome.

The deceased father of the proband was reported to have only SHFM, without cleft lip/palate or other features associated with the syndrome.

Blood samples were obtained with informed consent from the proband and her children. Genomic DNA was extracted from peripheral blood leukocytes using standard techniques. Initially, the DNA was amplified by PCR using primers that flank the entire coding sequence of the *TP63* gene. The amplified product was sequenced bi-directionally using the BigDye Terminator v3.1 Cycle Sequencing Kit method in an ABI 3730 DNA Analyzer equipment (Applied Biosystems, Foster City, CA, USA). Sequencing of both strands was performed at least twice for each sample.

The results were analyzed by the Bioedit program (Ibis Biosciences, Carlsbad, CA, USA). Prediction of the effect of the mutation was performed with the PolyPhen-2, SIFT and MutationTaster2 online programs. The protein alignment analysis was done using the Universal Protein Resource (Uniprot) catalog available online. The mutation was also searched in the databases of the 1000 Genomes and the NHLBI Exome Sequencing Project. The numbering of nucleotides and amino acids used in this report is in accordance with systematic nomenclature approved by the Human Genome Variation Society (HGVS).

Molecular analysis of the *TP63* gene identified a novel heterozygous missense change c.1037C > G in exon 8, present in the proband and in her affected son. It was absent in her unaffected daughter. This mutation has never been described before and is not present in the 1000 Genomes and NHLBI exome sequencing project databases. The mutation c.1037C > G in *TP63* that results in a p.Ala346Gly amino acid substitution, was predicted to be probably damaging by PolyPhen-2 (score of 0,997) and SIFT (score of 0), and to be disease causing by MutationTaster2. The protein sequence alignment showed that alanine at position 346 lies in the DBD region and is highly conserved across different species (Uniprot). The *TP63* mutation here described has been submitted to the ClinVar Database (SCV000189629). More than 30 different mutations have been described in the literature, and only two of them are located outside the DBD region (Celli *et al.*, 1999; Rinne *et al.*, 2006; Clements *et al.*, 2009).

Mutations in the DBD region were reported to disrupt the DNA-binding ability of p63, which results in reduced transactivation activity (Celli *et al.*, 1999). p63 protein plays an important role in the development, proliferation, and maintenance of stratified epithelia, such as the epidermis (Koster *et al.*, 2004). Mice lacking *TP63* display defects in epithelial lineage development and fail to develop stratified epithelia and epithelial appendages (Mills *et al.*, 1999; Yang *et al.*, 1999), which explains the skin, glands, teeth and hair defects in the EEC syndrome.

*Irf6*, one of p63 target genes, is needed for skin differentiation and palate development, including palate closure (Moretti *et al.*, 2010). Mutations in *Irf6* were associated



**Figure 2** - Images showing split-hand/foot malformation (SHFM) in patients investigated. (A) proband; (B) proband's son; (C) hands of proband; (D) hands of proband's son; (E) feet of proband.

with cleft lip/palate and other developmental disorders in mice (Richardson *et al.*, 2006). A feedback regulatory loop between *Irf6* and p63 was suggested (Guerrini *et al.*, 2011), and, therefore, mutations in *TP63* could explain orofacial cleft.

The apical ectodermal ridge (AER) on limb-bud, which is responsible for limb development, is maintained by self-renewing stem cells. It was speculated that p63 has an essential role in preserving self-renewal capacity of progenitor epithelial cells (Yang *et al.*, 1999). Moreover, there is evidence that p63 is able to induce the transcription of *Dlx5* and *Dlx6* promoters in the AER (Lo Iacono *et al.*, 2008), and the *DLX5* gene was already associated with SHFM (type I) in humans (Shamseldin *et al.*, 2012). Double knockout mice for *Dlx5* and *Dlx6* present SHFM (Merlo *et al.*, 2002). Restelli *et al.* (2014) proposed a model for a

regulatory loop during limb development. According to their model: p63 activates *Dlx5/Dlx6* transcription; *Dlx5/Dlx6* positively controls *Fgf8*; p63 also activates *Fgf8* transcription; Pin1 (peptidyl-prolyl *cis/trans* isomerase NIMA-interacting-1) induces p63 destabilization mediated by proteasome degradation; on the other hand, *Fgf8* acts on the AER cells and promotes p63 stability, apparently because it protects p63 from Pin-1 interaction. The activation of this loop and maintenance of p63 levels allows stratification and specialization of ectoderm cells in the AER. These findings, taken together, can explain the clinical features of isolated forms of SHFM and SHFM in EEC syndrome.

In defects associated to *TP63* mutations, genotype-phenotype-correlations are hampered by extremely variable expressivity both among and within families (Rinne *et al.*, 2007; Di Lorio *et al.*, 2012). The situation is further

complicated by the fact that affected individuals by (apparently) different diseases associated with the *TP63* gene may be carriers of identical mutations: such is the case of the mutation p.R243W associated with EEC (Celli *et al.*, 1999) and ADULT (Avitan-Hersh *et al.*, 2010) syndromes, and the case of the mutation p.R319H, that can cause the EEC syndrome (van Bokhoven *et al.*, 2001) as well as non-syndromic SHFM (Ianakiev *et al.*, 2000).

This variable expressivity of EEC is evident in the present study: besides the proband's son not having cleft lip/palate, features present in his mother, his leg malformation is much more severe when compared to the one presented by his mother. Since the proband's son does not have cleft lip/palate, his phenotype is similar to that of ADULT syndrome, which may not include clefting, breast hypoplasia or freckling (Berk *et al.*, 2012). The significant clinical overlap between the two phenotypes demonstrates that the distinctions between EEC and ADULT syndromes are somewhat artificial, as already pointed out by Reisler *et al.* (2006). The father of the proband allegedly presented only SHFM malformation affecting his four limbs, without other manifestations of EEC. This could indicate that the mutation c.1037C > G might also be associated with non-syndromic SHFM, in spite of a certain degree of uncertainty due to the fact that this individual is already deceased and could not be personally examined by one of us.

As already stressed, this is the first report of the mutation c.1037C > G (p.Ala346Gly). Clements *et al.* (2009) found a mutation at the same amino acid position, a change from alanine to asparagine (p. Ala346Asp) (in their report the mutation was formerly described as p.Ala307Asp), which caused the EEC syndrome in two patients of the same family, mother and son. They both displayed hypodontia and bilateral SHFM. The ectodermal anomalies were similar in mother and son. Some differences between the patients from his and our report were observed in the clefting pattern, since it was bilateral in the case of p.Ala346Asp mutation and unilateral in ours. Another important difference between these two reports concerns the large spectrum of clinical variability observed within families: while the patients studied by Clements *et al.* (2009) differed only in relation to the presence of lacrimal ducts dysplasia, our patients differed in relation to the presence of cleft lip/palate and to the variability spectrum of limb malformation.

The present findings contribute to elucidate the complex phenotype-genotype correlations in EEC syndrome and other related *TP63*-mutation syndromes. The possibility of the mutation c.1037C > G being related to both ADULT syndrome and SHFM is also raised by the findings here reported.

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## Internet Resources

- 1000 genomes, <http://www.1000genomes.org/> (accessed January 15, 2014).
- ClinVar, <http://www.ncbi.nlm.nih.gov/clinvar/intro/> (accessed October 9, 2014).
- Human Genome Variation Society (HGVS), <http://www.hgvs.org/mutnomen> (accessed October 15, 2013).
- MutationTaster2, <http://www.mutationtaster.org/> (accessed July 01, 2014).
- NHLBI Exome Sequencing Project (ESP): Exome Variant Server, <http://evs.gs.washington.edu/EVS/> (accessed July 01, 2014).
- OMIM: online mendelian inheritance in man, <http://www.omim.org/> (accessed January 15, 2014).
- PolyPhen-2: prediction of functional effects of human nsSNPs, <http://genetics.bwh.harvard.edu/pph2/> (accessed December 20, 2013).
- SIFT: sorting Tolerant From Intolerant, <http://sift.jcvi.org/> (accessed July 01, 2014).
- Uniprot: universal protein resource, <http://www.uniprot.org/> (accessed December 20, 2013).

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