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TP63 Gene Mutations in Chinese P63 Syndrome Patients

ABSTRACT

TP63 plays an essential role in the development of epidermis and skin appendages. Mutations in *TP63* can give rise to a series of syndromes characterized by various combinations of ectodermal dysplasia, limb malformations, and orofacial clefting in many populations. To test whether *TP63* is the disease-causative gene for these phenotypes in Chinese, we recruited two Chinese Ectrodactyly-Ectodermal-dysplasia-Cleft lip/palate syndrome (EEC) cases and a Limb-Mammary-Syndrome (LMS) patient to carry out *TP63* gene sequencing. Three missense mutation, c.812G>C (Ser271Thr), c.611G>A (Arg204Gln), and c.680G>A (Arg227Gln), which lead to the substitution of highly conserved amino acids in the DNA-binding domain of TP63, were identified. These mutations were predicted to disrupt DNA-binding specificity and affinity. To our knowledge, this is the first report of EEC and LMS syndromes in individuals of Chinese descent. Analysis of our data demonstrated that *TP63* is critical for the development of ectoderm in humans.

KEY WORDS: ectrodactyly, ectodermal dysplasia, and cleft lip/palate syndrome (EEC); limb-mammary syndrome (LMS); *TP63* mutation; protein modeling; DNA binding domain.

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INTRODUCTION

Ectrodactyly-Ectodermal-dysplasia-Clefting syndrome (EEC, OMIM 604292) is a rare autosomal-dominantly inherited condition characterized by (1) split hands and feet, (2) ectodermal dysplasia, and (3) cleft lip and/or cleft palate (Roelfsema and Cobben, 1996). Additional EEC features include lacrimal duct anomalies, urogenital defects, conductive hearing loss, chronic respiratory infections, ventricular cardiomyopathy, and developmental delay. Limb-Mammary syndrome (LMS, OMIM 603543) is an EEC-like syndrome with two major symptoms: ectrodactyly, and hypoplastic mammary glands and nipples. In contrast to EEC, LMS patients rarely have hair and skin involvement, and if facial clefting is present, it is always limited to the palate (van Bokhoven *et al*., 1999, 2001).

Most cases (> 80%) of classic EEC and LMS are caused by *TP63* gene mutations (Celli *et al*., 1999; van Bokhoven *et al*., 1999; Kosaki *et al*., 2001; Rinne *et al*., 2006, 2007). Mutations of *TP63* are also responsible for the following syndromic malformations, Ankyloblepharon-Ectodermal dysplasia-Clefting (AEC, OMIM 106260), Acro-Dermato-Ungual-Lacrimal-Tooth (ADULT, OMIM 103285, an LMS-like syndrome with ectrodactyly, and mammary hypoplasia plus excessive freckling, but without facial clefting), and Rapp-Hodgkin ectodermal dysplasia (RHS, OMIM 129400, an AEC-like syndrome with characteristic midfacial hypoplasia) (McGrath *et al*., 2001; van Bokhoven *et al*., 2001; Barrow *et al*., 2002; Duijf *et al*., 2002; Dianzani *et al*., 2003; Kantaputra *et al*., 2003; Chan *et al*., 2004; Kannu *et al.,* 2006; Rinne *et al*., 2006, 2007). In addition, two non-syndromic human disorders are associated with *TP63* mutations: isolated split hand/foot malformation type IV (SHFM-IV, OMIM 605289) (Ianakiev *et al*., 2000) and, recently, non-syndromic cleft lip/palate (Leoyklang *et al*., 2006). The term "*TP63* syndromes" is therefore suggested for these related disorders featured by overlapping phenotype spectrums. Analysis of large amounts of data has demonstrated distinct *TP63* mutation patterns and locations for each of these syndromes (Rinne *et al*., 2006). The apparent genotype-phenotype relationship is, in part, attributed to the various biochemical structures of *TP63*.

TP63, located on chromosome 3q27, encodes a transcription factor homologous to the tumor suppressors *p53* and *p73* (Ianakiev *et al*., 2000; van Bokhoven *et al*., 2002; Bertola *et al*., 2004; Mikkola, 2007). Genomic organization of *TP63* is complexed with at least 6 different isoforms. It may express from 2 different 5′ transcriptional start sites that generate 2 major proteins: TATP63 or ΔNTP63, which contains or lacks the N-terminal transactivation domain. In addition, alternative splicing at the 3′ ends of the transcripts generates 3 C-termini (α, β, and γ). The longest isoform, TA-TP63α, has all functional domains: transactivation domain, DNA binding domain, tetramerization domain, sterile alpha motif, and

Table 1. Clinical Manifestations of EEC and LMS Patients Tested for *TP63* Gene Mutations in This Study*

Patient ID	Sex	Age (yrs)	Clinical Manifestations: Limb Abnormality	Ectodermal Dysplasia	Hypodontia	Facial Clefting	Mammary Glands	Diagnosis
1	M	10	Yes	Yes	14,16,17,21,22,23,24,25,26,27, 31, 32,33,34,36,37,42,43,44,46,47	Yes	Normal	EEC
2	M	2	Yes	Yes	51,54,61,64,72,74,82,84	Yes	Normal	EEC
3	F	13	Yes	Yes	13,17,23,27,33,37,43,47	No	Abnormal	LMS

* See Appendix Table for detailed clinical features of the EEC and LMS patients in this study.

transactivation inhibitory domains (Van Bokhoven *et al*., 2002; Brunner *et al*., 2002b; Keyes *et al*., 2006). Although the predominant role of *TP63* in the morphogenesis of skin and its appendages is obvious, the molecular pathways regulated by *TP63* are still emerging (Yang *et al*., 1999; McGrath *et al*., 2001). In this study, we investigated the molecular basis of EEC and LMS in three patients of Chinese origin to further examine the possibility of allelic heterogeneity.

MATERIALS & METHODS

Participants and Sample Collection

Three unrelated probands were recruited during routine dental clinic examinations and by interviews with their family members. Diagnoses of EEC and LMS were based on medical history, clinical examinations, and evaluations of radiographs. An EEC patient should have at least two of the three major features (described in the 'Introduction'). An LMS patient should fulfill the EEC criteria; however, the clefting is of the palate alone; hair and skin are excluded from ectodermal involvement, and there is definitive mammary gland and nipple aplasia or hypoplasia (van Bokhoven *et al*., 1999). All procedures were performed in accordance with an Institutional Review Board protocol approved by the Hospital and School of Stomatology, Wuhan University. After informed consent was obtained, venous blood samples were drawn from all available participants. Genomic DNA was extracted from whole-blood leukocytes or Epstein-Barr virus-transformed B-lymphoblastoid cell lines by standard proteinase-K phenol chloroform methods.

Mutation Screening

Specific primers were designed to amplify the coding exons and intron-exon boundaries (primers available on request) with the use of Primer 3 online software. Amplified fragments were purified with a polymerase chain-reaction (PCR) purification kit (Omega, Norcross, GA, USA). DNA sequences were obtained from both strands with an ABI PRISM-3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA). Mutations co-segregating with the phenotype were verified in 250 unrelated healthy control individuals by direct sequencing and, where possible, by restriction enzyme digestion. The position of the mutation corresponds to the original published TA-TP63α sequence (GenBank accession no. AF075430) (Celli *et al*., 1999), which does not include the 39 additional codons for the amino-terminal end that were reported later (GenBank accession AF091627).

Protein 3D Modeling

Modeling of the TP63 protein 3D structure was based on its homologue, p53, for which the crystal structure has been solved (Cho *et al*., 1994). We performed the modeling by using Swiss-PdbViewer v4.00 software. References for the TP63 DNA-binding domain structure model are available at www.cmbi.kun.nl/gv/service/TP63dimer (Duijf *et al*., 2002).

RESULTS

Clinical Findings

Both of the two EEC probands had classic features, including ectodermal dysplasia, ectrodactyly, and orofacial clefts (Figs. 1A, 1B, Appendix Fig., Table, Appendix Table). Of interest, the female proband (II:2) of family 3 had severe hypodontia (at least 6 permanent teeth absent), mild features of ectodermal dysplasia, and limb abnormalities restricted to her feet (Fig. 1C, Appendix Fig., Table, Appendix Table). The patient's mammary glands were underdeveloped. Her nipples were much smaller than normal. In addition, she had 2 small accessory nipples: one on the left side and the other on the right side. Severe nipple anomalies and the absence of cleft lip/palate confirmed her diagnosis of LMS. However, we did not take any photographs of the hypoplastic mammary gland, because her parents would not consent. The severities and types of limb deformities between these patients were variable, ranging from bilateral split hands and feet of missing phalanges to absent metacarpals and metatarsals, with or without syndactyly and webbing. The absence of ankyloblepharon filiform, face deformity, and freckling excluded AEC, RHS, and ADULT from all these patients. Their family members were clinically healthy.

Mutation Analysis

Direct sequencing of the *TP63* gene revealed 3 different nucleotide changes in the unrelated Chinese families. Two mutations were recurrent, and one was novel. In patient 1, we detected an

AGC to ACC (c. 812 G>C) transversion at codon 271 in exon 7 (Fig. 2). This missense mutation is expected to cause a p.Ser271Thr substitution in the TP63 protein. In family 2, the patient carried a c.611G>A transition (Fig. 2), leading to a predicted amino acid residue change from Arg to Gln at codon 204 (p.Arg204Gln). This missense mutation has been reported previously in European families (Celli *et al*., 1999). In patient 3, we identified a CGA to CAA (c.680G>A) transition at codon 227 with a predicted substitution p. Arg227Gln of a highly conserved amino acid (Fig. 2). Co-segregation of the mutant alleles with the phenotype was confirmed in all families. None of the mutations was detected in their parents, siblings, or in 250 healthy control individuals, and no other nucleotide changes were identified. Paternity was confirmed by means of polymorphic genetic markers as described previously (Ye *et al*., 2005; Fan *et al*., 2008), indicating that mutations found in these patients were *de novo*.

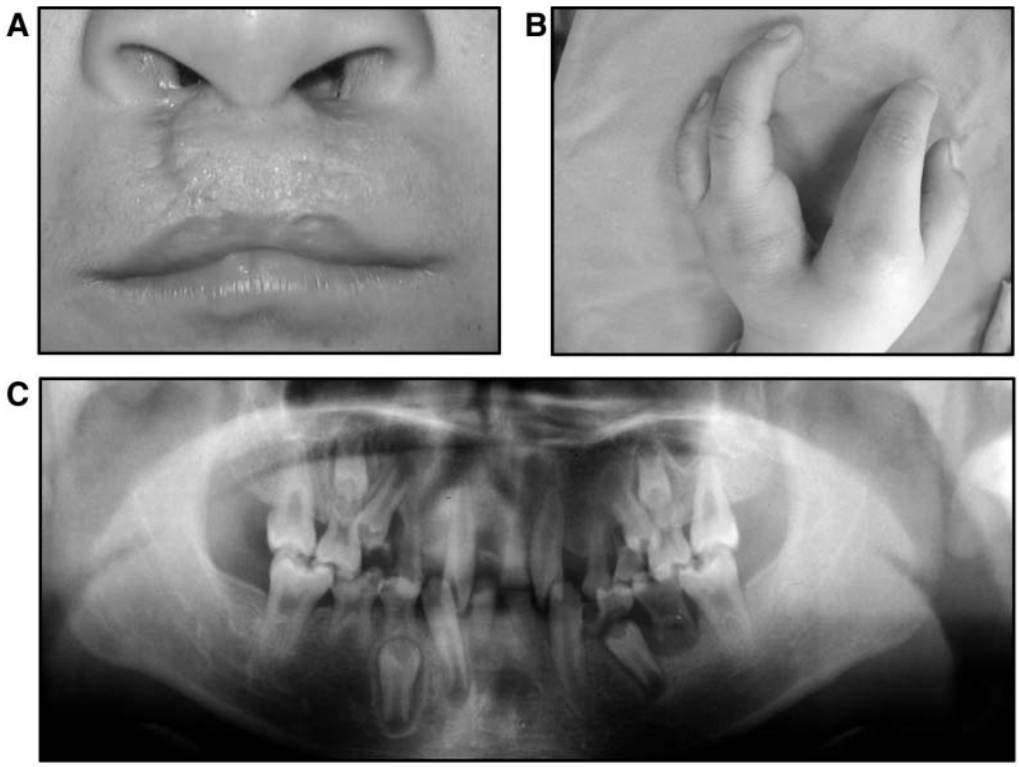

Figure 1. Typical clinical manifestations of the three affected subjects with EEC and LMS. **(A)** Patient 1 has bilateral complete cleft lip (repaired) and cleft palate. **(B)** Typical "lobster-claw" malformations on both hands and feet in patient 2. Note syndactyly of both toes with dystrophic nails. **(C)** Panoramic radiograph displayed multiple permanent teeth missing from both maxillary and mandibular arches in the 13-year-old girl (patient 3).

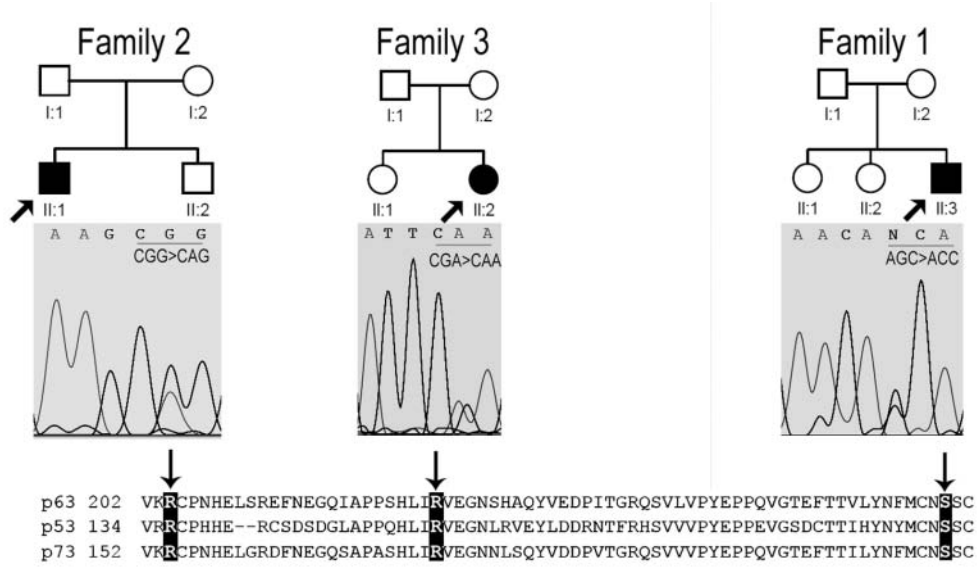

Figure 2. Pedigree structures and DNA-sequencing chromatograms of the mutations. Filled symbols represent affected individuals and open symbols unaffected individuals. Squares represent males, and circles represent females. Alignment of the amino acid sequence between the DNA-binding domains of P53, P63, and P73 demonstrates extreme conservation of all mutated codons. Arrows indicate the mutation sites.

Protein 3D Modeling

All *TP63* mutations identified in this study were in the DNA-binding domain of the molecule. According to the structure model, it can be deduced that the *TP63* assumes a loop-sheet-helix motif and 2 large loops that make up the DNA-binding surface of the protein. Modeling of the TP63 3D structure suggests that Arg204 and Ser271 residues were localized on the outer surface of the crystal structure (Figs. 3A, 3B). These residues occupy a space in proximity to the DNA double-helix chains, which suggests that they would partially or completely abolish the binding capacity between TP63 and the DNA molecule. The residue Arg227 lies neither on the DNA-binding surface nor in the area involved in its dimerization

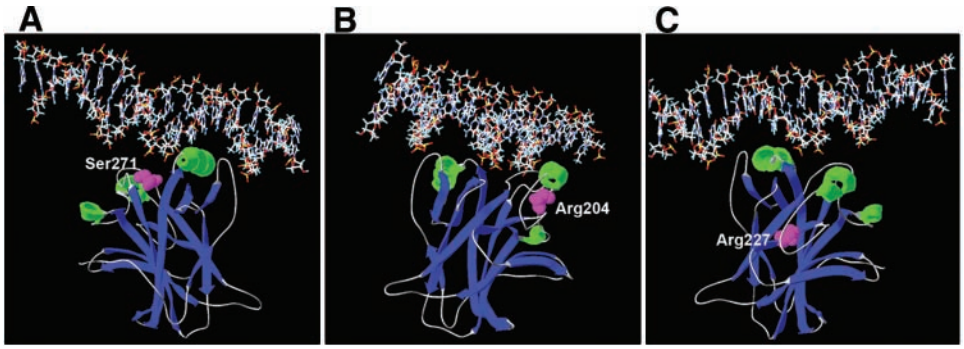

Figure 3. Model of the DNA-binding domain of TP63, based on the structure of its homologue, p53. Protein is shown as a ribbon model with blue strands and helices, green turns, and loops, respectively. The side-chains of interesting amino acid residues mutated in EEC (Arg204Gln and Ser271Thr) and LMS (Arg227Gln) are shown in pink as molecular surface models. DNA is represented as a stick model. A color version of this Fig. can be seen in the online version of the article. **(A, B)** Arg204Gln and Ser271Thr (highlighted) mutations found in two EEC patients in the region of the DNA-binding domain that is close to the DNA. **(C)** The Arg227Gln residue mutated in the LMS patient was found outside the DNA-binding surface.

(Fig. 3C). Thus, Arg227Gln might disrupt the integrity or stability of the TP63 protein DNA-binding domain structure rather than affect the DNA-binding capacity.

DISCUSSION

Here we describe for the first time 3 *TP63* mutations in Chinese individuals, including 2 mutations that have been reported elsewhere in other populations (Celli *et al*., 1999). The Arg204Gln, Ser271Thr, and Arg227Gln mutations are concluded to be pathogenic for the following reasons: (1) The substitution occurred within the DNA-binding domain and is predicted to change amino acids highly conserved in TP63, and molecular modeling data supported these results; (2) these mutations were not identified in either their healthy family members or a cohort of 250 normal control individuals, and the possibility of a rare polymorphism is very low; and (3) TP63 Arg204 and Arg227 have been considered to be mutation hotspots in other EEC populations. Our results further confirmed that LMS and EEC are allelic disorders, and *de novo* mutations in the *TP63*-related syndromes are not very rare (van Bokhoven *et al*., 1999; Brunner *et al*., 2002a). The evident *TP63* mutational homogeneity may be explained by the fact that the 2 codons are located at CpG sites of mutational hotspots. Another possibility is that this mutational profile is caused by a specific pathogenetic gain-of-function mechanism, as was previously suggested (Brunner *et al*., 2002b).

So far, up to 31 *TP63* mutations have been found in EEC cases. All are missense mutations clustered in the DNA-binding domain (exon 5-7) except for a single frameshift mutation occurring in the SAM domain (exon 13) in a patient with EEC phenotype as well as mammary gland hypoplasia (Brunner *et al*., 2002b). The 5 arginine codons (Arg204, Arg227, Arg279, Arg280, Arg304) are the most frequently mutated residues, covering nearly 80% of EEC cases, all of them located in the CpG islands (van Bokhoven *et al*., 2002). In patient 1 with EEC, a novel *de novo* mutation Ser271Thr was detected. We noticed that 2 other *de novo* mutations of nearby amino acids have been reported to be associated with the EEC syndrome (Ser272Asn, Cys273Tyr). S272N was found in a Dutch patient manifesting with ectrodactyly and syndactyly, cleft lip and palate, hypodontia, sparse hair, and lacrimal duct abnormality (Celli *et al*., 1999; van Bokhoven *et al*., 2001). These observations support the hypothesis that mutations involving the DNA-binding domain in the *TP63* will lead to EEC syndrome. The change of amino acid Agr204, a mutation hotspot located at the beginning of the DNA-binding domain, causes a phenotype that is consistent with EEC. We found the Arg204Gln mutation in patient 2 with classic EEC phenotype, indicating that the Arg204 hotspot might be as common in Chinese subjects as described in other populations.

Amino acid Arg227 is the only *TP63* mutation hotspot that does not have a homologous *p53* mutation hotspot as well (Celli *et al*., 1999). Previous studies have shown that approximately 40% of EEC patients present both cleft lip and palate, whereas Arg227 patients seldom have orofacial clefting and limb defects. To date, only two cases have been reported to have cleft palate only, and syndactyly was completely absent in Arg227 mutation patients (Duijf *et al*., 2002). In contrast, genito-urinary abnormalities are frequently observed in Arg227 mutation cases (van Bokhoven *et al*., 2001). We reported here the first case harboring an Arg227Gln mutation with ectrodactyly and syndactyly in the feet and without clefts. These characteristics suggest that Arg227 mutation might exert specific effects different from those of other hotspot codons. EEC and LMS were previously considered as distinct clinical entities, and genotype-phenotype analysis revealed that only those affecting specific amino acids in the DNA-binding domain will yield an EEC phenotype (Barrow *et al*., 2002; Brunner *et al*., 2002b; Rinne *et al*., 2006, 2007). However, patient 3 in our study exhibited an LMS-like phenotype (absence of clefting, skin, hair, and lacrimal duct anomalies, and a consistent feature of mammary gland and/or nipple hypoplasia with 2 or 3 accessory nipples), despite a typical EEC Arg227Gln mutation manifestation. Several examples have shown that the same *TP63* mutation can lead to different clinical conditions (Kantaputra *et al*., 2003; Bertola *et al*., 2004). LMS and ADULT syndrome seem to be caused by the same mutation, Gly134Asp, as well as EEC syndrome and non-syndromic cleft lip/palate due to the Arg313Gly mutation (Duijf *et al*., 2002). The reason for clinical variability within EEC and LMS is unclear and may be secondary to other genetic factors, such as modifier genes (Mills *et al*., 1999; Ray *et al*., 2004). The complex genotype-phenotype correlation we observed highlights the difficulty of a purely clinical diagnosis. More functional studies of the Arg227Gln mutation should be carried out to address the phenotype-genotype relation in more detail.

In conclusion, we confirmed that *TP63* mutations are associated with EEC and LMS in Chinese patients. Thus, the spectrum of malformation diseases due to *TP63* abnormalities is further extended. Our findings further underlie not only the clinical overlapping of *TP63*-related syndromes, but also the difficulty of establishing unequivocal genotype-phenotype correlations. Further studies in larger numbers of patients and families with EEC and EEC-like syndromes are needed to elucidate these issues.

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