Heterozygous Mutation in the SAM Domain of p63 Underlies Rapp-Hodgkin Ectodermal Dysplasia

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ABSTRACT

Several ectodermal dysplasia syndromes, including Ectrodactyly-Ectodermal dysplasia-Clefting (EEC) and Ankyloblepharon-Ectodermal Dysplasia-Clefting (AEC) syndromes, are known to result from mutations in the *p63* gene. We investigated whether Rapp-Hodgkin syndrome (RHS) is also caused by mutations in the *p63* gene. We identified a heterozygous *de novo* germline missense mutation, S545P, in the sterile-alpha-motif (SAM) domain of p63, in a Thai patient affected with RHS. This is the first genetic abnormality to be described in RHS. The amino acid substitution is the most downstream missense mutation in *p63* reported thus far. Histological assessment of a skin biopsy from the patient's palm showed hyperkeratosis and keratinocyte cell-cell detachment in the upper layers of the epidermis, along with numerous apoptotic keratinocytes. Collectively, these investigations demonstrate that RHS is also caused by mutations in *p63* and that the clinical similarities to AEC syndrome are paralleled by the nature of the inherent mutation.

KEY WORDS: AEC syndrome, dental anomalies, palmoplantar keratoderma, *p63* gene, Rapp-Hodgkin syndrome.

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INTRODUCTION

Rapp-Hodgkin syndrome (RHS), or Rapp-Hodgkin ectodermal dysplasia, was first described over 30 years ago in an affected mother, son, and daughter with a combination of anhidrotic ectodermal dysplasia, cleft lip, and cleft palate (Rapp and Hodgkin, 1968). The clinical syndrome is comprised of a characteristic facies (narrow nose and small mouth), wiry, slow-growing, and uncombable hair, sparse eyelashes and eyebrows, obstructed lacrimal puncta/epiphora, bilateral stenosis of external auditory canals, microsomia, hypodontia, cone-shaped incisors, enamel hypoplasia, dystrophic nails, and cleft lip/cleft palate. Approximately 45 cases of this developmental disorder, usually with autosomal-dominant inheritance, have been reported (Summitt and Hiatt, 1971; Wannarachue *et al*., 1972; Stasiowska *et al*., 1981; Silengo *et al*., 1982; Salinas and Montes, 1988; Rodini *et al*., 1990; Santos *et al*., 1990; Breslau-Siderius *et al*., 1991; Walpole and Goldblatt, 1991; Kantaputra *et al*., 1998; Neilson *et al*., 2002), thus defining RHS as a discrete clinical entity (Online Mendelian Inheritance in Man [OMIM] no. 129400).

Nevertheless, RHS does display some clinical overlap with other ectodermal dysplasia syndromes, notably Ectrodactyly-Ectodermal Dysplasia-Clefting (EEC) syndrome (OMIM 129900) and Ankyloblepharon-Ectodermal Dysplasia-Clefting (AEC), also known as Hay-Wells syndrome (Hay-Wells Syndrome, OMIM 106260) (van Bokhoven and McKeon, 2002). Differentiating features of AEC and RHS appear to be the presence of ankyloblepharon in AEC and microsomia in RHS, but making an accurate diagnosis on clinical grounds alone may be difficult. Indeed, this was highlighted in a case of a woman with RHS whose son had features of EEC syndrome (Moerman and Fryns, 1996). The affected child also had ankyloblepharon, a component of AEC syndrome. Other overlap syndromes have also been reported (Cambiaghi *et al*., 1994; Rowan, 1996), suggesting that perhaps there might be a common genetic pathology to many of these and other related ectodermal dysplasia syndromes. It is noteworthy that some parents with AEC syndrome had affected children without ankyloblepharon. Furthermore, the ankyloblepharon of some affected individuals is so subtle or friable at birth that it often goes unrecognized, and these cases may subsequently be diagnosed as RHS.

The molecular basis of most cases of EEC syndrome has recently been shown to involve mutations in the *p63* gene (Celli *et al*., 1999). The majority of mutations is comprised of heterozygous missense changes in the DNA-binding domain of p63 (van Bokhoven *et al*., 2001). Moreover, AEC syndrome has subsequently been reported to result from missense mutations in *p63*, specifically within the sterile alpha motif (SAM) (McGrath *et al*., 2001). Thus, both these disorders are caused by heterozygous germline mutations in p63, and a genotype-phenotype correlation for the site of the mutation (DNA-binding domain or SAM domain) has been defined. To date, no pathogenetic mutations in *p63* have been reported in patients with RHS. Therefore, in this study we investigated the molecular basis of RHS to further examine the possibility of allelic heterogeneity.

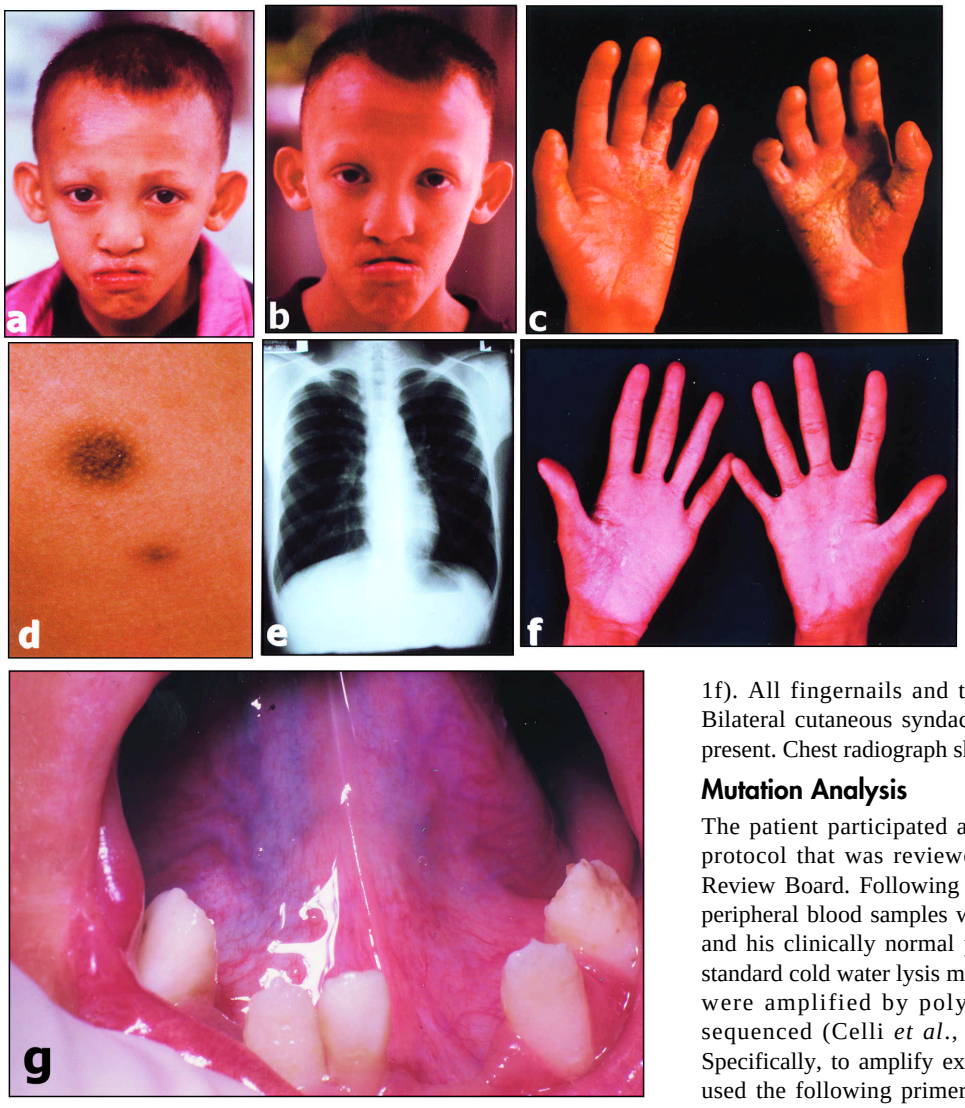

Figure 1. Clinical pictures of the patient. **(a)** Patient at 8 yrs old. Note repaired cleft lip and palate, microsomia, sparse hair, and diffuse dermatitis of the skin. **(b)** Patient at 14 yrs old. **(c)** Keratoderma of palms at 8 yrs. **(d)** Supernumerary nipple. **(e)** Hypoplastic scapulae. **(f)** Keratoderma of palms at 14 yrs. Note improvement of the condition as he aged. **(g)** Congenitally missing mandibular left central incisor. Enamel hypoplasia of the mandibular right lateral incisor and left canine.

MATERIALS & METHODS

Clinical Details

A 14-year-old Thai boy was seen in The Department of Pediatric Dentistry, Faculty of Dentistry, Chiang Mai University. He had been diagnosed and reported previously as having Rapp-Hodgkin syndrome (Kantaputra *et al*., 1998) (Figs. 1a, 1b). His clinical findings included characteristic facies, slow-growing and uncombable hair, sparse eyebrows and eyelashes, obstructed lacrimal puncta and epiphora, microsomia, repaired cleft lip and cleft palate (Figs. 1a, 1b), taurodontism, multiple caries, unerupted premolar, glossy tongue, hypodontia, enamel hypoplasia, and congenital absence of the lingual frenum and sublingual caruncles, including submandibular and sublingual salivary gland duct openings (Fig. 1g) (Kantaputra *et al*., 1998). However, the abnormal scalp hair lacked pili canaliculi, a feature noted in some other cases of RHS (Salinas and Montes, 1988). The ears protruded, with evidence of bilateral bony external auditory canal stenoses, and he had a moderate mixed pattern of hearing loss. Hypoplastic and supernumerary nipples were observed (Fig. 1d). The skin was very dry, with some hypopigmented areas. Diffuse dermatitis of the scalp, face, and forearms was present (Figs. 1a, 1b). Palms and soles demonstrated keratoderma with multiple fissures, although the degree of keratoderma had improved when he became a teenager (Figs. 1c, 1f). All fingernails and toenails were dystrophic and ridged. Bilateral cutaneous syndactyly of the second and third toes was present. Chest radiograph showed hypoplastic scapulae (Fig. 1e).

Mutation Analysis

The patient participated after providing informed consent to a protocol that was reviewed and approved by the Institutional Review Board. Following informed consent given by his father, peripheral blood samples were taken from the affected individual and his clinically normal parents, and DNA was extracted by a standard cold water lysis method. Individual exons of the *p63* gene were amplified by polymerase chain-reaction (PCR) and sequenced (Celli *et al*., 1999; van Bokhoven *et al*., 2001). Specifically, to amplify exon 13 and flanking introns of *p63*, we used the following primers: forward primer 5′-CTT ATC TCG CCA ATG CAG TTG G-3′, and reverse primer 5′-AAC TAC AAG GCG GTT GTC ATC AG-3′. The expected PCR product size was 241 base pairs (bp). For PCR amplification, 250 ng of genomic DNA was used as the template in an amplification buffer containing 6.25 pmol of the primers, 37.5 nmol $MgCl_2$, 5 mmol of each nucleotide triphosphate, and 1.25 U *Taq* polymerase (Applied Biosystems, Warrington, UK) in a total volume of 25 μL in an OmniGene thermal cycler (Hybaid, Basingstoke, UK). The amplification conditions were 94ºC for 5 min, followed by 38 cycles of 94ºC for 45 sec, 53ºC for 45 sec, and 72ºC for 45 sec. Aliquots (5 μL) of the PCR products were analyzed by 2% agarose gel electrophoresis. PCR products were then sequenced directly with the use of Big Dye labeling in an ABI 310 genetic analyzer (Applied Biosystems). Potential mutation was verified by restriction endonuclease digestion or by direct sequencing (forward and reverse) and assessed in 300 control chromosomes to exclude non-pathogenetic sequence variants.

Skin Biopsy

Following informed consent, a 4-mm punch biopsy was taken from the patient's palm while he was under general anesthetic (given for dental surgery). Skin was fixed in 10% formalin and processed for routine light microscopy with paraffin-embedding. Five-micron sections were stained with hematoxylin and eosin and photographed.

RESULTS

Mutation Analysis

Sequencing of the PCR products spanning exon 13 with the use of DNA from the affected individual revealed a heterozygous T>C point mutation at nucleotide position 1633 within exon 13 (numbering based on the originally published TA-p63α sequence [Yang *et al.*, 1998], GenBank accession no. AF075430). The mutation converted a serine residue (TCC) to proline (CCC) and was designated S545P (Fig. 3). This mutation has not been reported previously in patients with EEC, AEC, or other *p63*-related disorders and was not detected in direct sequencing of DNA from this patient's father or from 150 control subjects. No other heterozygous or homozygous sequence variants for the remainder of the *p63* gene were found in the patient's DNA.

Histological Findings

Light-microscopic examination of palmar skin showed acanthosis and hyperkeratosis with a mild upper dermal interstitial chronic inflammatory cell infiltration and some exocytosis of lymphocytes throughout the upper epidermis. Most notably, in the upper spinous layers of the epidermis there was evidence of abnormal keratinocyte differentiation with numerous apoptotic keratinocytes, acantholysis, and disruption of the granular layer (Fig. 2).

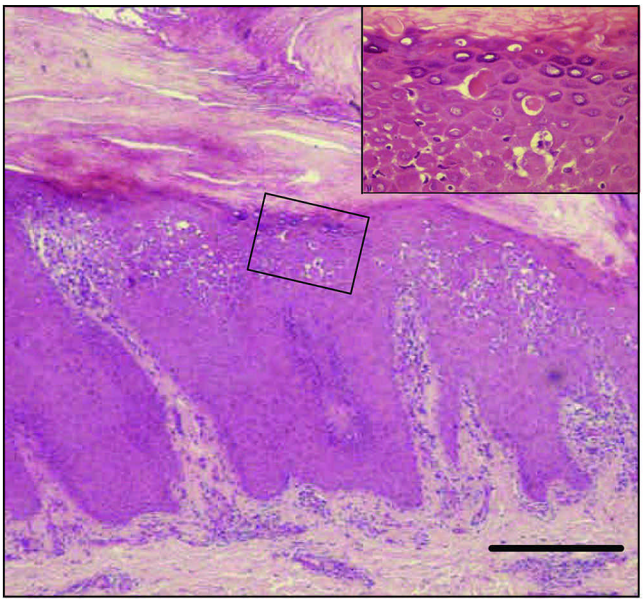

Figure 2. Histology of palmar skin shows acanthosis and hyperkeratosis. There is a mild upper dermal interstitial chronic inflammatory cell infiltrate and exocytosis of lymphocytes within the superficial dermis. Most notably, in the upper spinous cell layers there are numerous apoptotic keratinocytes (seen at higher magnification in the inset) with keratinocyte separation (acantholysis) and disruption of the granular cell layer. Bar = 100 microns.

DISCUSSION

In this study, we have elucidated the molecular basis of RHS in one affected individual. Our findings provide evidence of further allelic heterogeneity for mutations in *p63* and the diverse clinical phenotypes therein. Indeed, we can now provide molecular data to support the earlier reports of clinical overlap between EEC syndrome, AEC syndrome, and RHS (Cambiaghi *et al.*, 1994; Moerman and Fryns, 1996; Rowan, 1996). RHS is clinically most similar to AEC syndrome, and this is borne out at a molecular level. In AEC syndrome, 8 heterozygous germline missense mutations in the SAM domain of p63 have been reported, all of which are clustered within the first 3 of 5 helical domains (McGrath *et al.*, 2001). In contrast, the missense mutation in our case of RHS occurs in the fourth helix of the SAM domain, albeit just 4 amino acids downstream from the most 3′ of the AEC mutations. This raises the fundamental question of whether this subtle difference is sufficient to justify labeling AEC syndrome and RHS as separate entities. To begin to answer this, it is clear that further patients with a clinical diagnosis of RHS will need to be screened for *p63* mutations. Interestingly, recent mutation analysis in one other RHS family (with mixed cleft type) did not reveal any *p63* mutations (Neilson *et al.*, 2002). Locus heterogeneity of RHS therefore cannot be ruled out, further adding to the complexity of attempts to unravel the molecular basis of this ectodermal dysplasia syndrome. From a clinical perspective, we believe that our patient has RHS rather than AEC syndrome because of the presence of the characteristic facies, microsomia, obstructed lacrimal puncta/epiphora, and palmoplantar keratoderma and the absence of ankyloblepharon. At a molecular level, it is necessary to be more circumspect, since little is currently known about the function of the SAM domain other than its potential as a site for protein-protein interactions (Thanos and Bowie, 1999). Characterization of the roles of individual amino acids in these interactions will be necessary to define more specific functions. However, attempts to define genotype-phenotype correlation for *p63*-mutations in ectodermal dysplasia syndromes are further complicated by reported diverse phenotypes arising from identical amino acid substitutions. For example, mutations in arginine 280 may give rise to EEC syndrome, split hand-split foot malformation, or, rarely, no detectable abnormalities (van Bokhoven *et al.*, 2001). Likewise, mutations in arginine 304 have been shown to underlie a spectrum of EEC syndrome phenotypes (Hamada *et al.*, 2002). Clearly, there are other influences on the phenotypic consequences of particular mutations in the TP63 transcription factor, and therefore a simple genotype-phenotype correlation based on a specific amino acid substitution is fundamentally flawed. Until the nature of these additional modifying factors or the full spectrum of inherent gene pathology becomes apparent, we believe that it is still more appropriate to retain the descriptive eponyms such as RHS, which is the most appropriate diagnosis that encompasses the clinical features present in our patient.

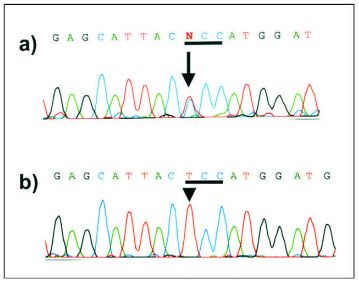

Figure 3. Nucleotide sequencing of *p63* exon 13 from normal **(b)** and the affected patient **(a)** shows a heterozygous T>C point mutation that converts a serine residue (TCC) to proline (CCC), designated as S545P.

In addition to disclosing the initial *p63* gene mutation in RHS, our study reports the first histological assessment of the erosive palm- and sole-skin thickening that is a characteristic feature of RHS. TP63 is known to have an important role in the regulation of epidermal stem cell proliferation (Schultz *et al*., 1997; Mills *et al*., 1999; Parsa *et al*., 1999; Yang *et al*., 1999; de Laurenzi *et al*., 2000), and its expression in keratinocytes has been used to distinguish stem cells from transit amplifying cells and other keratinocytes (Pellegrini *et al*., 2001). The missense mutation S545P in the p63 SAM domain clearly perturbs normal epidermal differentiation and maturation, leading to acanthosis (thickening) and hyperkeratosis (increased scale) as well as focal impairment of keratinocyte cell-cell attachment and, most notably, numerous apoptotic keratinocytes (colloid bodies). TP63 is known to influence keratinocyte apoptosis (Yang *et al*., 1998) in response to certain stimuli such as ultraviolet irradiation (Liefer *et al*., 2000) and in response to knockout mutations (Flores *et al*., 2002), but inhibition of apoptosis appears to be abrogated in the presence of the heterozygous missense mutation S545P. Analysis of these clinico-pathological data may therefore provide insight into one of the functions of the p63 SAM domain in normal epithelial physiology. Specifically, our case demonstrates the first example of increased epidermal apoptosis in association with a human germline mutation in *p63*. This is clearly distinct from AEC syndrome, where no increase of apoptosis is observed (McGrath *et al*., 2001). Palmoplantar keratoderma and mixed hearing loss, found in our patient, have not been reported in other patients affected with RHS (van Bokhoven and Brunner, 2002). The novel finding of hypoplastic scapula might have been related to *p63* mutation, or it might have been a coincidence.

The *p63* mutation detected may also be relevant to our understanding of the dental pathology present in some ectodermal dysplasia syndromes. The present patient has unerupted premolar and taurodontism, *i.e.*, large dental pulp and short roots, and although there are no previous reports of *p63* expression in dental pulps, the findings in our patient give credence to the significance of *p63* expression during dental development, since large pulp chambers are known to occur as a consequence of defective dentin formation in the dental pulp, a key role for normally functioning odontoblasts. This process is evidently disrupted in the RHS patient described here. The SAM domain mutation S545P, and its effects on oral mucosal development, may also be relevant to the pathogenesis of the patient's glossy tongue and absence of lingual frenum and of sublingual caruncles, since these are the rare clinical features in other *p63*-related syndromes.

In summary, this case discloses the molecular basis of RHS and provides new clinico-pathological insight into the consequences of a specific p63 SAM domain mutation on epithelial development.

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