CHAPTER IV

DISCUSSION

4.1 *TP63* mutation and syndromic hypodontia with/without orofacial clefts

4.1.1 A novel R227P mutation of TP63 gene in a Thai family with EEC syndrome

Among the total of 30 cases studied, we detected a novel R227P (+/-c.R227P) missense mutation of the TP63 gene in a Thai family with EEC syndrome, consisting of affected girl (CGL DNA number 181) and her affected father (CGL DNA number 182). This novel R227P mutation has not been reported before.

Ectrodactyly-Ectodermal dysplasia-Cleft lip/palate (EEC) syndrome is characterized by a deep median cleft of the hands and feet, missing digits (ectrodactyly, also known as split hand/split foot malformation: SHFM); with one or more features of ectodermal defects, which may present as defects of hair, skin, nails, teeth, nasolacrimal ducts, and exocrine glands, such as sweat, salivary, sebaceous, and mammary glands; and cleft lip with or without cleft palate. EEC syndrome is well known for having both variable clinical expression and incompleted penetrance (Roelfsema and Cobben, 1996). Interfamilial variability appears to be significantly larger than intrafamilial variability, suggesting that more than one gene or allele might be involved or specific modifier effects, possibly through interacting genes (Brunner et al., 2002; Duijf et al., 2003; Roelfsema and Cobben, 1996; Sifakis et al., 2001).

Interestingly, clinical variability is a hallmark of EEC syndrome, with clinical expression ranging from severe abnormality to clinically normal (Brunner et al., 2002; Buss, 1994; Buss et al., 1995; Celli et al., 1999).

The affected structures of patients with EEC syndrome can be related to the structures that express high levels of p63 during embryogenesis. *p63* is expressed in several ectoderm-derived tissues: it is essential for the initiation of the epithelial stratification program during embryonic development (Barbieri and Pietenpol, 2006; Koster and Roop, 2004a; Laurikkala et al., 2006; Mills et al., 1999; Yang et al., 1999). *p63* is normally expressed in the ectodermal surfaces of the limb buds, branchial arches and epidermal derivatives (which develop as a result of epithelial-mesenchymal interactions), such as hair follicles, whiskers, teeth and exocrine glands (which developed from adjacent epithelial and neural crest-derived mesenchymal tissues, including mammary, sweat, prostate, salivary, and lacrimal glands) (Mills et al., 1999; Yang et al., 1998; Yang et al., 1999).

The structural defects found in EEC Syndrome can be evident in p63 Knockout Mice. Mice lacking all p63 isoforms exhibit severe developmental defects including limb truncations, epidermal defects and craniofacial anomalies. The epidermis is thin, and fails to stratify and lacks ectodermal appendages such as hairs, teeth and several glands, including mammary, salivary and lacrimal glands (Mills et al., 1999; Yang et al., 1999). Comparison of the phenotype of patients who have EEC syndrome with that of $p63^{-/-}$ mice (Mills et al., 1999; Yang et al., 1999) results in a strikingly similar pattern of involved structures.

4.1.1.1 Phenotype of a Thai family with EEC syndrome with the R227P mutation

The affected Thai girl with the R227P mutation showed the phenotype of classic EEC syndrome, including ectrodactyly, ectodermal defects and cleft lip with palate, whereas her affected father with the R227P mutation showed only ectrodactyly of the right hand and ectodermal defects. The EEC phenotype of this Thai family with EEC syndrome with the R227P mutation has demonstrated intrafamilial variability, including abnormalities of limbs, ectodermal defects, and orofacial clefts. The phenotype will be discussed as follows:

4.1.1.1.1 Abnormalities of Limbs

The affected girl with the R227P mutation had ectrodactyly of both hands and the right foot, and syndactyly of the right 4th and 5th toes, whereas her affected father who with the same mutation had ectrodactyly of the right hand, bifid right thumb, flexion contracture of the distal phalanx of the left index finger, and small and narrow 2nd toes. The severity of limb defects is highly variable.

Regarding the p63 and the development of the limbs, p63 plays a crucial role in apical ectodermal ridge (AER) development (Yang et al., 1999). p63 is a developmental component in signaling pathways, which is required for normal limb morphogenesis. *p63* is normally expressed in the AER, a specialized pseudo-stratified epithelium surrounding the limb bud (Mills et al., 1999; Yang et al., 1999). The AER is crucial for the formation and identity of digits. It regulates the pattern of

the developing limb bud along the proximal-distal axis (shoulder-finger direction) (Duijf et al., 2003). The AER formation is induced by mesodermal signaling to the overlying ectoderm. Thus, in the event that p63 is absent, epithelial-mesenchymal signaling fails to develop in the limb bud as demonstrated by limb defects found in $p63^{-/-}$ embryos (Duijf et al., 2003). It has been demonstrated that AER is absent in the developing limb bud of $p63^{-/-}$ embryos. $p63^{-/-}$ mice had complete absence of the hindlimbs and severely truncated forelimbs. It is possible that p63 plays a greater role in the development of the hindlimbs than of the forelimbs (Koster and Roop, 2004b; Mills et al., 1999; Yang et al., 1999). In conclusion, the limb defects/truncations are due to a abnormal formation or maintenance of the AER (Barbieri and Pietenpol, 2006; Koster and Roop, 2004b; Laurikkala et al., 2006; Lo Iacono et al., 2008; Mills et al., 1999; Yang et al., 1999).

Ectrodactyly (ectro-, from the Greek: abortion and –dactyly, from the Greek: finger, toe) is a limb malformation affecting the distal portion of the hand and/or foot, and may present with deep median clefts of the hand and/or foot, syndactyly, and aplasia and/or hypoplasia of the phalanges, metacarpals, and metatarsals. For example, mildly affected patients may have only syndactyly, while the hands and feet may have a lobster claw-like appearance in the more severe cases (Buss, 1994; Duijf et al., 2003; Sifakis et al., 2001). Ectrodactyly is a consistent feature of EEC syndrome. The ectrodactyly develops as a result of a failure to maintain median AER activity, either through increased cell death, or reduced cell proliferation, resulting in the defects in the median AER. The anterior and posterior AER are less severely affected. This explanation appears to suit the relatively normal development of the anterior (thumb) and posterior (the 5th finger) digits. In addition, there are a number

of key molecules in the signaling pathways of the developing limb bud that may interact with p63, including FGFs, BMPs, WNT signaling molecules, and homeobox-containing proteins, such as MSX1 and MSX2 (Duijf et al., 2003) (Figure 4.1).

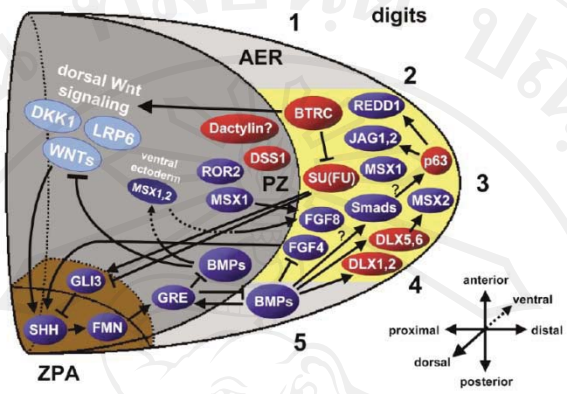


Figure 4.1 Signaling pathways in the failure to maintain median AER, or in defective AER signaling, underlie SHFM. The positions of the AER (light grey represents anterior and posterior AER while yellow represents median AER), underlying progress zone (PZ; dark grey), and zone of polarizing activity (ZPA; brown) are indicated. Numbers 1–5 refer to the future positions of digits 1–5, respectively (reproduced from Duijf et al., 2003).

The severity of the defects of hands and feet is highly variable. The clinical variability not only exists between patients, but also between limbs of a single individual (Duijf et al., 2003; Sifakis et al., 2001). Asymmetry of the abnormalities between the right and the left sides is common (Celli et al., 1999; Duijf et al., 2003). Buss has previously reported 24 cases of EEC, consisting of distal limb defects from simple syndactyly to tetramelic cleft hand and foot (Buss et al., 1995). Moreover It has been described that some individuals with EEC syndrome have only nonspecific limb abnormalities, such as clinodactyly of fifth fingers, ulnar deviation of third

fingers, abortive mesoaxial polydactyly of the hand, hypoplastic fifth ray of the foot, or normal limbs (Maas et al., 1996), syndactyly of fingers (Barrow et al., 2002; Celli et al., 1999), shortening of, or missing, digits (Barrow et al., 2002).

4.1.1.1.2 Ectodermal defects

The affected girl with the R227P mutation had a number of ectodermal defects, including dry and sparse scalp hair, slightly dry skin, and thin nails. Her affected father had normal hair, dry skin, normal fingernails, and hypoplastic toenails. In addition, he had oral manifestations, including congenital absence of the permanent mandibular canines, generalized microdontia, prominent marginal ridges of the permanent maxillary incisors, round-shaped permanent molars, barrel-shaped permanent maxillary central incisors, enamel hypoplasia of the permanent mandibular first premolars, and extensive dental caries. Abnormalities of nasolacrimal ducts, and exocrine glands such as sweat, salivary, sebaceous, and mammary glands were unremarkable in this Thai family with EEC syndrome.

Ectodermal defects are one of the three main characteristics of the TP63-associated syndromes. They manifest as the abnormal development/growth of tissues and structures that are developed from ectoderm. In this condition, the development of skin, hair, teeth, nails and several exocrine glands, such as sweat and sebaceous glands, are frequently abnormal. The epidermis of these patients can be very dry, itchy, and hypopigmented. In the severely affected patients, widespread areas of the skin might be eroded. The amount of scalp and body hair is often reduced and hair can be wiry or curly. The eyelashes and eyebrows may be absent. The number of

teeth is often reduced, indicating that there is a reduced number of tooth placodes. Teeth can also be malformed by a conical shape and poor enamel formation, causing subsequent dental caries. Nails can be dystrophic. The absence or paucity of sweat glands is reported, leading to diminished perspiration, which can be life-threatening. The development and function of sebaceous and salivary glands are frequently abnormal. Lacrimal duct defects and obstruction of the lacrimal ducts have been reported. In addition aplasia/hypoplasia of mammary glands/nipples are not uncommon in patients with EEC syndrome (Rinne et al., 2007).

p63 is essential for the development of ectodermal appendages, including hair, skin, nails, teeth, nasolacrimal ducts, and exocrine glands, including mammary, sweat, prostate, salivary, and lacrimal glands, which develop from epithelial-mesenchymal interactions. *p63* is normally expressed in the ectodermal surfaces of the limb buds, branchial arches, and epidermal appendages (Mills et al., 1999; Yang et al., 1998; Yang et al., 1999).

The severity and type of the ectodermal defects also are highly variable, both within and among families. In addition, the characteristics of ectodermal defects have been described to be quite variable between TP63-associated syndromes. Only some individuals have demonstrated defects in all of the ectodermal structures (Rinne et al., 2007).

In this part of the discussion of ectodermal defects, each major affected structure will be discussed individually, as follows:

A. Hair

This Thai family with EEC syndrome with the R227P mutation had dry and sparse dark hair. The scalp hair, eyebrows, and/or eyelashes are affected in nearly all EEC cases, with the scalp hair generally being coarse and dry, sparse, and slow-growing. However, axillary hair in males and females, and facial hair in males are usually normal (Bamshad, 2008). Hair/whisker follicles are completely absent in p63 knockout mice (Mills et al., 1999; Yang et al., 1999). Thus p63 is required in the ectoderm for the formation of hair. During hair follicle and vibrissae development, *p63* expression is detected throughout the epithelium, (Laurikkala et al., 2006; Rinne et al., 2007) where p63 has been colocalized with K5 and Ki67, which are epithelial markers for differentiation (Rufini et al., 2006).

There have been studies of hair using electron microscopy in patients with TP63-asoociated syndrome. Pili torti (twisting of hair shafts) and pili analiculi (canal-like depressions along the axis) have been reported in patients with Rapp-Hodgkin syndrome (Chan et al., 2005; Dianzani et al., 2003; Sahin et al., 2004). The SEM study of hair of this Thai family with EEC syndrome demonstrated small hair bulbs, thin hair shafts, and hypoplastic cuticles. It is noteworthy that the SEM of hair of patients with other TP63-associated syndromes should also be analyzed.

B. Skin

p63 is known to be essential for development of epidermis. p63 is required either for the commitment to stratification of skin or maintenance of epidermal stem

cells (Mills et al., 1999; Yang et al., 1999). During the development of epidermis, *p63* is strongly expressed in the basal, or progenitor, cells of several epithelial structures (Yang et al., 1998). Skin of $p63^{-/-}$ mice has striking defects, including a single layer of epithelium in the skin, as found by Mills et al. and the presence of patches of differentiated keratinocytes, as found by Yang et al. (Mills et al., 1999; Yang et al., 1999) The skin phenotype has been described to be the result of either a lack of commitment of the immature ectoderm to epidermal lineages (Mills et al., 1999), or a lack of proliferative potential of the p63-deficient epidermal stem cells (Yang et al., 1999).

The dry skin found in this Thai family with EEC syndrome could be the result of defects of sebaceous glands, which provide moisture and lubrication for the skin. p63 is essential for sebaceous gland formation. *p63* also is expressed in the ectodermal surface of the sebaceous glands (Mills et al., 1999; Yang et al., 1999).

C. Teeth

The dental anomalies found in this Thai family with EEC syndrome consist of hypodontia, malformed teeth, enamel hypoplasia, and extensive dental caries. p63 plays crucial roles in tooth development (Laurikkala et al., 2006). $p63^{-/-}$ mice have no teeth (Mills et al., 1999; Yang et al., 1999); their tooth development ceases at the dental lamina stage. *p63* is normally expressed in teeth, which develop as a result of epithelial-mesenchymal interactions (Mills et al., 1999; Yang et al., 1999). The study of p63 mRNA expression during tooth morphogenesis has shown that ΔNp63

isoforms are highly detected in embryonic ectoderm at all stages of tooth development. Interestingly, TAp63 isoforms are not detected at all at any stages of tooth development (Laurikkala et al., 2006). As p63 is known to be crucial for tooth development, which originates from epithelial-mesenchymal interaction during development, it is not surprising that hypodontia is one of the most common findings in patients with *T*P63-associated mutations (Barrow et al., 2002; Chan et al., 2004; Chan et al., 2005; Dianzani et al., 2003; Hamada et al., 2002; Neilson et al., 2002; Pozo et al., 2004; Reisler et al., 2006; Rinne et al., 2008; van Bokhoven et al., 2001). Enamel hypoplasia is also a common feature in a large number of patients with TP63-associated syndromes (Chan et al., 2004; Chan et al., 2005; Dianzani et al., 2003; Hamada et al., 2002; Kantaputra et al., 1998; Reisler et al., 2006; Sahin et al., 2004; van Bokhoven et al., 2001). This might be linked to the DLX3 gene, which is a downstream target of p63 (Radoja et al., 2007). Both Dlx3 and p63 are essential for development of the epidermis and/or embryonic appendages (Koster and Roop, 2004b; Morasso and Radoja, 2005; Radoja et al., 2007). The DLX3 plays a crucial role in enamel formation and its mutations can cause Tricho-Dento-Osseous syndrome (TDO; MIM 190320) (Price et al., 1998a), which is characterized by curly hair, enamel hypoplasia, taurodontism, and a thick cortical bone (Lee et al., 2008). In addition, extensive dental caries (Chan et al., 2005; Pozo et al., 2004; Wessagowit et al., 2000) has frequently been described as a finding in TP63-associated syndromes. Other dental anomalies which have been reported to be associated with TP63-associated syndromes are delayed dental eruption (Barrow et al., 2002), and malformed teeth (Barrow et al., 2002; Chan et al., 2005; Dianzani et al., 2003; Neilson et al., 2002; Pozo et al., 2004; Reisler et al., 2006; Sahin et al., 2004). The

literature describing the association between the dental phenotype and TP63-associated syndromes is negligible. It is suggested that dental phenotype be documented in publications related to TP63 mutations.

D. Nails

This Thai family with EEC syndrome presented with thin and hypoplastic toenails. The defects of nails have been reported in nearly all individuals in almost all families with EEC syndrome (van Bokhoven et al., 2001). Defects of nails have also been reported in other syndromes (Kantaputra et al., 2003; Leoyklang et al., 2006; Pozo et al., 2004). There have not been studies of the roles of p63 and nail formation.

E. Other ectodermal structures: Nasolacrimal ducts and exocrine glands, such as sweat, salivary, sebaceous, and mammary glands

p63 is essential for the formation of nasolacrimal ducts and exocrine glands, such as sweat, salivary, sebaceous, and mammary glands. *p63* is expressed in the ectodermal surfaces of these structures (Mills et al., 1999; Yang et al., 1999). The exocrine glands, including mammary, lacrimal and salivary glands, are absent in $p63^{-/-}$ mice (Mills et al., 1999; Yang et al., 1999). However, the abnormalities of nasolacrimal ducts, and exocrine glands, such as sweat, salivary, sebaceous, and mammary glands, were unremarkable in this Thai EEC family with R227P mutation, whereas other families with *TP63*-associated syndrome with the R227Q mutation had defects in these organs, especially lacrimal duct abnormalities and

aplastic/hypoplastic mammary glands/nipples. Besides reduced saliva secretion, reduced axillary sweating and dry skin have been reported (Maas et al., 1996; Maclean et al., 2007; O'Quinn et al., 1998; Reisler et al., 2006; van Bokhoven et al., 2001). Aplastic/hypoplastic mammary glands/nipples have been found in some patients with EEC syndrome with other mutations such as R204Q, R279H, 1689InsA, 1693-1694DelTT, 1860-1861Del AA (van Bokhoven et al., 2001). Supernumerary nipples, which develop additionally along the mammary line (two stripes of pseudostratified ventral surface ectoderm) (Mikkola, 2007), also are observed in patients with other TP63-associated syndromes, such as EEC syndrome with the R279Q mutation (van Bokhoven et al., 2001), AEC syndrome with the T533P mutation (McGrath et al., 2001) and RHS with the S541P mutation (Kantaputra et al., 2003). In $p63^{-/-}$ mice, expression of *Lef-1*, which is an early mammary placode marker, is absent in the ectoderm overlying the sites at which mammary buds normally form (Mills et al., 1999).

4.1.1.1.3 Orofacial clefts

The affected girl had left cleft lip and a complete cleft of the secondary palate, which was typical for EEC syndrome. Her father did not have any kind of any orofacial clefts. Interestingly, cleft lip with/without palate is found in approximately 40% of patients with EEC syndrome (Rinne et al., 2007). Cleft palate has been reported in about 30% of patients with Limb-Mammary Syndrome (LMS). In patients with AEC syndrome, cleft lip with/without palate or cleft palate alone are observed, 44% and 80%, respectively. Orofacial clefts have not been reported in patients with

ADULT syndrome (Rinne et al., 2007). All EEC patients have typical facies, whether or not possessing severe ectodermal defects of cleft lip/palate. These facial features include a broad nasal root, bridge, and tip, and flared ala nasi. These midface/palatal abnormalities are however usually milder in EEC patients without cleft lip/palate (Bamshad, 2008).

The position of this novel R227P mutation in a Thai family with EEC syndrome is at arginine (R) 227. R227 has been known to be a hot spot for *TP63* mutation (Brunner et al., 2002; van Bokhoven et al., 2001; van Bokhoven and Brunner, 2002; van Bokhoven and McKeon, 2002). The R227Q mutation has been described in the largest previously reported family with EEC syndrome. Micturition difficulties and extensive dental caries were common features found in that Dutch family with EEC syndrome (Maas et al., 1996; van Bokhoven et al., 2001) (Figure 4.2).

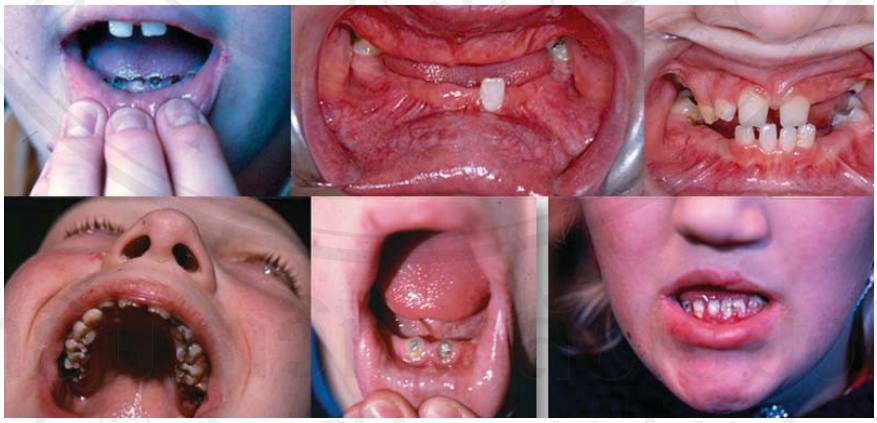


Figure 4.2 Some affected individuals of the largest previously reported family with EEC syndrome with the R227Q mutation are reported to have unusually extensive dental caries (Courtesy of Professor Dr. Raoul CM Hennekam, London, England).

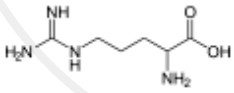
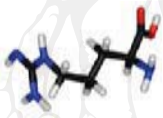
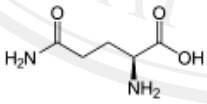
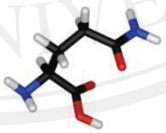
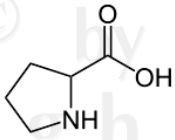
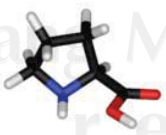
4.1.1.2. R227P mutation: mutation hotspot and highly conserved amino acid

Mutation analysis of both father and daughter revealed a heterozygous G>C at nucleotide position 680 within exon 6 (+/-c.680 G>C). This mutation changed an amino acid from arginine (CGA) to proline (CCA) at position 227 (p.R227P), which is located in the DNA-binding domain (DBD) of *TP63*. A mutation at the R227 position has been found in six previously reported families (Maas et al., 1996; Maclean et al., 2007; O'Quinn et al., 1998; Reisler et al., 2006; van Bokhoven et al., 2001). Arginine (R), a polar-basic side chain amino acid, is substituted by glutamine (Q), a polar-neutral side chain amino acid (p.R277Q) (van Bokhoven et al., 2001). This novel R227P mutation (p.R227P) caused the amino acid change from arginine (R) to proline (P), a non polar-neutral side chain amino acid that forms a cyclic five-membered ring and that lacks the backbone amide proton (Strachan and Read, 2004). The protein structures/models of the wild type, R227P and R227Q are demonstrated **Table 4.1**.

The R227 amino acid is a mutation hotspot. The arginine (R) codon 227, which was mutated in several unrelated patients, is crucial for direct interactions with DNA target sequences, and its mutation is highly harmful to DNA binding and transactivation activity (van Bokhoven and Brunner, 2002; van Bokhoven and McKeon, 2002). An explanation is possible for a mutation to occur at this location, which is highly specific for *TP63* mutations. It is more likely that the restricted mutation spectrum in EEC syndrome might reflect a specific pathogenetic mechanism. This possibility is supported by the finding that some different missense mutations, at amino acids R204W/Q, R279C/H/O, R280C/H/S, and R304W/Q, give

rise to EEC syndrome (Brunner et al., 2002; van Bokhoven et al., 2001; van Bokhoven and Brunner, 2002; van Bokhoven and McKeon, 2002). Although mutations can occur at many different sites along the TP63 gene, only those affecting specific amino acids in the DNA binding domain of the molecule will yield an EEC phenotype (Brunner et al., 2002).

Table 4.1 Comparison between 3 amino acids; arginine (R), glutamine (Q), proline (P) (pictures from http://en.wikipedia.org/wiki/Amino_acid)

Amino acid	**Chemical structure**	**Model**	**Side chain polarity**	**Side chain charge (pH 7)**
Arginine (R)			Polar	Positive
Glutamine (Q)			Polar	Neutral
Proline (P)			Non-polar	Neutral

4.1.1.3 Structural model of p63 protein affected with R227P mutation

The structural model of the DNA binding domain predicts that R227 is located in the central β-strand (S4) of the five-stranded antiparallel β-sheet and forms hydrogen bonds through its amide proton and its carboxyl oxygen with T327, located in the adjacent β-strand (S9). The R227P mutation removes one hydrogen bond within this important central β-sheet and destabilizes the structure of the DNA binding domain. The effect of the R227Q mutation, however, cannot be explained by the destabilization of the DNA binding domain since the side chain of R227 is not involved in intra-molecular salt bridges or hydrogen bonds. Influencing the interaction with binding partners is a more likely the explanation since a charged side chain is replaced with a polar but neutral one. Influencing intermolecular interactions is also a likely mechanism for the R227P mutation, in addition to the destabilization of the DNA binding domain. However for both mutations, it is unlikely that they affect the DNA binding affinity (made by Professor Volker Dötsch, Frankfurt/Main, Germany) **(Figure 4.3)**.

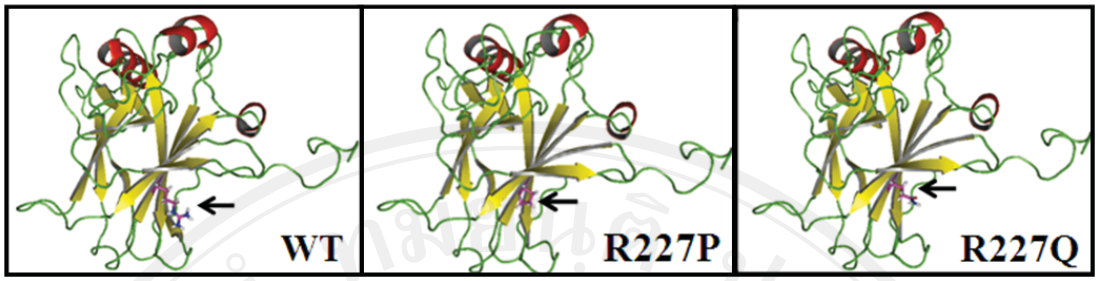


Figure 4.3 Comparing structural models of p63 DNA-Binding Domain (DBD) between WT, R227P mutation, and R227Q mutation (black arrow). The R227P mutation can destabilize the structure of the DBD because proline, that forms a cyclic five-membered ring and that lacks the backbone amide proton, removes one hydrogen bond within this important central β-sheet of DBD, while the R227Q mutation cannot destabilize the DBD because the side chain is not involved in intra-molecular salt bridges or hydrogen bonds (Courtesy of Professor Volker Dötsch, Frankfurt/Main, Germany).

4.1.1.4 Comparison of R227Q mutations and this R227P mutation

The R227Q mutation has been reported in five families with EEC syndrome (Maas et al., 1996; Maclean et al., 2007; O'Quinn et al., 1998; van Bokhoven et al., 2001)and in a family with ADULT syndrome (Reisler et al., 2006). The phenotypes of the previously reported families with the R227Q mutation and that of this family with the R227P mutation were compared (Table 4.2).

This Thai family with EEC syndrome with the R227P mutation had extensive dental caries but had no micturition difficulties. In contrast, the striking phenotypes of the largest family with EEC syndrome with the R227Q mutation were micturition difficulties and extensive dental caries. It is of interest that five of the six families reported with the R227Q mutation had micturition difficulties and this was not found in these patients with EEC syndrome. Of the 30 members of the families with the R227Q mutation, at least 24 had micturition difficulties. This finding has demonstrated that micturition difficulties were quite common in individuals with the R227Q mutation. This may be caused by the specific difference of changed amino acids. However, it is impossible to make a conclusion from these two patients with the R227P mutation. Micturition difficulties have also been described in a patient with the R279H mutation (van Bokhoven et al., 2001).

Table 4.2: Reviewing of the clinical features of R227Q mutations in EEC & ADULT syndrome and Comparing with R227P mutation in the Thai family with EEC syndrome

	van Bokhoven et al., 2001			Reisler et al., 2006	Maclean et al., 2007		Thai family with EEC syndrome
	Mass et al., 1996 1 family (14 individuals) EEC Syndrome	O'Quinn et al., 1998 1 family (6 individuals) EEC Syndrome	van Bokhoven et al., 2001 1 family (2 individuals) EEC Syndrome	1 family (2 individuals) ADULT Syndrome	2 families (4 individuals &2 individuals) EEC Syndrome Family A (4)	EEC Syndrome Family B (2)	1 family (2 individuals) EEC Syndrome
Mutation	**R227Q**	**R227Q**	**R227Q**	**R227Q**	**R227Q**		**R227P**
Ectrodactyly	+	+	+	-	+	-	+
Syndactyly	+	-	-	+	-	-	+
Trichodysplasia	+	+	*NM*	+	+	+	+
Dry skin	+	+	*NM*	*NM*	+	-	+
Freckling skin	*NM*	*NM*	*NM*	+	*NM*	*NM*	*NM*
Onychodysplasia	+	+	*NM*	+	+	+	+
Hypodontia	+	+	+	+	+	-	+
Dental anomalies	*NM*	*NM*	*NM*	+	*NM*	+	+
Lacrimal ducts abnormalities	+	+	+	+	+	+	-
Reduced saliva secretion	*NM*	*NM*	*NM*	+	*NM*	*NM*	-
Reduced axillary sweating	+	*NM*	*NM*	+	-	-	-
aplasia/hypoplasia of mammary gland/nipple	+	+	*NM*	+	+	+	-
CL/P	+	-	-	-	-	-	+
CP	-	-	-	-	+	-	-
Genitourinary anomalies	+	+	*NM*	+	+	-	-
Bladder problems	+	*NM*	*NM*	+	+	+	-
Micturition difficulties	+	+	*NM*	+	+	+	-

Note: +, present; -, absent; *NM*, not mentioned

4.1.1.4.1 p63 and micturition difficulties

The bladder forms during embryogenesis; the cloacal cavity at the posterior end of the embryo is partitioned by the uro-rectal septum into the ventral urogenital sinus (UGS) and the dorsal hindgut. The UGS subsequently develops into the urethra, bladder and urachus. The UGS epithelium differentiates into a stratified transitional epithelium, known as urothelium, whereas the mesenchyme of the UGS differentiates into the lamina propria and the smooth muscle of the bladder, known as the detrusor muscle. Interaction between the UGS epithelium and its mesenchyme (epithelial-mesenchymal interaction) is crucial for the development of the bladder (Cheng et al., 2006), as previous studies have shown that UGS epithelium provides key signaling input that promotes differentiation of the UGS mesenchyme into smooth muscle. Therefore, in the absence of bladder epithelium, bladder smooth muscles do not develop normally (Baskin et al., 1996). This may partly be responsible for the micturition difficulties in patients with the R227Q mutation. It has been described that p63 is not required for the formation of bladder epithelium but it has an essential role in the differentiation of a transitional urothelium (Mills et al., 1999; Urist et al., 2002; Yang et al., 1999). TAp63 is normally detected in the basal cells of the bladder urothelium, but ΔNp63 is absent or weakly detected in these cells (Comperat et al., 2006; Di Como et al., 2002; Park et al., 2000; Yang et al., 1998). ΔNp63γ and ΔNp63β have been found to be the predominant isoforms expressed in bladder epithelium during development. ΔNp63 has been described as playing an important anti-apoptotic role during bladder development and failure of this role might lead to bladder carcinogenesis (Cheng et al., 2006). Besides, elevated expression of ΔNp63 and decreased expression of TAp63 has been identified in bladder cancer as well

(Comperat et al., 2006). Another study has shown that p63 is regulated in bladder carcinogenesis and that *p63* expression is lost in most invasive cancers, whereas papillary superficial tumors maintain *p63* expression (Urist et al., 2002).

4.1.1.4.2 p63 and extensive dental caries

The extensive dental caries found in the affected father with the R227P mutation and in the largest previously reported family with EEC syndrome with the R227Q mutation is an interesting phenotype. Besides the micturition difficulties, the extensive dental caries has been described as an interesting finding in TP63-associated syndromes (Chan et al., 2005; Pozo et al., 2004; Wessagowit et al., 2000). TP63 should be considered as a candidate gene for high caries risk. Possibly either enamel hypoplasia, or a salivary abnormality resulting from abnormal salivary glands, or a combination of these, can result in the high risk of dental caries in TP63-associated syndromes.

A. Enamel hypoplasia

Enamel hypoplasia might be linked to the Dlx3, which is a downstream target of p63 (Radoja et al., 2007). Both Dlx3 and p63 are essential for development of the epidermis and/or embryonic appendages (Koster and Roop, 2004a; Morasso and Radoja, 2005; Radoja et al., 2007). The DLX3 plays a crucial role in enamel formation (Price et al., 1998a). Expression of *Dlx3* and *p63* are detected in the teeth (Hassan et al., 2004; Morasso et al., 1995; Morasso et al., 1999). In early murine tooth formation, at bud and cap stages, *Dlx3* is primarily expressed in the neural crest-derived mesenchymal component of the tooth. Its expression produces the dentin and

pulp at later stages. Interestingly, at late bell stage, when the inner enamel epithelium and mesenchyme undergo terminal differentiation, *Dlx3* expression shifts to being predominantly in the inner enamel epithelium and preameloblasts, while there is no *Dlx3*expression in the outer enamel epithelium. The inner enamel epithelium gives rise to the ameloblasts, which are responsible for enamel formation. Besides, the inner enamel epithelium eventually forms Hertwig's root sheath, which is responsible for establishing root morphology. Therefore, DLX3 mutation may disrupt the normal enamel and root formation, known as enamel hypoplasia and taurodontism , as seen in TDO syndrome (Price et al., 1998a). Moreover, the transcription of DLX3 is abrogated by mutations in the SAM domain of *TP63* that are associated with AEC syndrome (Radoja et al., 2007).

B. Defects of saliva

p63 plays crucial roles in salivary gland formation. *p63* is normally expressed in the ectodermal surfaces of salivary glands, which originate from epithelium-mesenchymal interactions. The salivary glands also are absent in the p63 knockout mouse (Yang et al., 1999). The defects of saliva may result from defects either of salivary gland structure or of salivary composition. This may be a consequence of xerostomia, which is a reduced production of saliva and leads to extensive dental caries (Bamshad, 2008). Chronic xerostomia is characterized by distribution of severe dental caries and oral mucosal changes (Daniels et al., 1975; van der Reijden et al., 1996). Subjects with impaired salivary flow rate often show high caries incidence (Spak et al., 1994).

4.1.2 **The absence** of *TP63* mutation in other 8 cases of syndromic hypodontia with/without orofacial clefts

TP63 mutation was not detected in other patients with syndromic hypodontia with/without orofacial clefts in this study. This result may be related to the small sample size in this study. Therefore increasing the sample size may increase a chance to detect the *TP63* mutation.

4.2 TP63 mutation and non-syndromic hypodontia, non-syndromic orofacial clefts

There was no TP63 mutation in patients with non-syndromic hypodontia or non-syndromic orofacial clefts in this study. This result may be related to the following assumptions:

1. The sample size in this study was too small. The mutation might have been found if the study had included more patients with variety of phenotypes.

2. Genes other than TP63 were responsible for these phenotypes.

Hypodontia is one of the most common findings in patients with TP63-associated syndromes (Barrow et al., 2002; Chan et al., 2004; Chan et al., 2005; Dianzani et al., 2003; Hamada et al., 2002; Neilson et al., 2002; Pozo et al., 2004; Reisler et al., 2006; Rinne et al., 2008; van Bokhoven et al., 2001). *TP63* mutation analysis in patients with non-syndromic hypodontia has not been previously performed. Therefore, this study performed *TP63* mutation analysis in patients with non-syndromic hypodontia to identify the *TP63* mutation. Unfortunately, mutations were not found in the coding

exons of *TP63*. They might have been found in *TP63* in the patients with non-syndromic hypodontia if more patients had been included in the study.

There have been a few studies about *TP63* mutations causing non-syndromic orofacial clefts. A previous study failed to find *TP63* mutations in among 31 white and 31 Filipino patients with non-syndromic orofacial clefts (Barrow et al., 2002). There has been only one case of non-syndromic orofacial cleft that is known to be associated with a mutation in *TP63*. The affected individual was a Thai and had a R313G mutation (Leoyklang et al., 2006).

4.3 Single nucleotide polymorphism (SNPs) of *TP63* in this study

All single nucleotide polymorphisms (SNPs) found in all samples have been reported before as follows:

Of 10 patients with non-syndromic hypodontia, 8 patients had RefSNP ID: rs1345186; dbSNP Database, a greater frequency than was found for any of the eight other SNPs identified.

Of 10 patients with non-syndromic orofacial clefts, 8 patients had RefSNP ID: rs62702062; dbSNP Database, a greater frequency than was found for any of the six other SNPs identified.

Of 10 patients with syndromic hypodontia with/without orofacial clefts, 9 patients had RefSNP ID: rs1345186; dbSNP Database, a greater frequency than was found for any of the eight other SNPs identified.

However in this study, sample size, which was only 30 patients, was too small to identify the relationship between phenotype and single nucleotide polymorphism. Therefore, sample size for future studies should have increased.