CHAPTER V

DISCUSSION

Family I

The author has identified a novel heterozygous missense c.721C>T (p.P241S) mutation in *MSX1* in a Thai patient affected with bilateral cleft lip and palate, hypodontia of left and right maxillary permanent lateral incisors, hypodontia of the left maxillary permanent second premolar, hypodontia of the right mandibular permanent third molar, and preaxial-polydactyly of the left thumb. This mutation was also found in his father, who had hypodontia of the right mandibular permanent lateral incisor, and in his brother, who had hypodontia of the right and left mandibular permanent third molars, and microdontia and dens invaginatus of left maxillary permanent lateral incisor. This *MSX1* variant might be responsible for syndromic orofacial clefts and non-syndromic hypodontia in this family, based on the following reasons.

First, *MSX1* is important for tooth and palatal development. Several studies have suggested that the *MSX1* mutations are associated with Witkop syndrome, non-syndromic orofacial clefts and non-syndromic hypodontia (Chishti et al., 2006; Jezewski et al., 2003; Kim et al., 2006; Lidral and Reising, 2002; Mostowska et al., 2006; Pawlowska et al., 2009; Suzuki et al., 2004; Tongkobpetch et al., 2006; van den Boogaard et al., 2000; Vastardis et al., 1996; Xuan et al., 2008). The similarity of phenotypes in this family and those in previous studies may be one reason to support

the hypothesis that p.P241S might be responsible for syndromic orofacial clefts and non-syndromic hypodontia in this family.

Second, *MSX1* is an especially strong candidate gene associated with cleft palate, maxillary hypoplasia and a failure of tooth development in the knockout mouse (Satokata and Maas, 1994). These phenotypes may serve to predict the phenotype observed in humans carrying *MSX1* mutations. From the association between *Msx1* mutation and phenotypes in this knockout study, *MSX1* mutation may also be the most possible genetic etiology in our case.

Third, p.P241S was not present in 200 chromosomes from 100 unaffected controls with the same ethnic background as the subjects, confirming that this mutation is not a single nucleotide polymorphism (SNP).

Fourth, this missense mutation (p.P241S) is located in the highly conserved region, the C-terminal domain of MSX1 protein, just downstream of the homeodomain (Finnerty et al., 2009). The ClustalX amino acid homology quality score revealed that this proline is conserved in the zebrafish, African clawed frog, chicken, house mouse, brown rat, cow, dog and chimpanzee. The conservation of this protein among various species indicates its important role in living organisms. Moreover, there is a substantial change in amino acid class, from non-polar proline to polar serine (Figure 5.1). This substitution may alter normal MSX1 protein function, but this hypothesis should be confirmed by functional analysis.

An interesting aspect of the phenotype in this report is the present of cleft lip and palate, missing teeth and microdontia in the same family. A previous study of a Dutch family with tooth agenesis and various combinations of CL/P and CPO in the same family also showed a nonsense mutation in *MSX1* (van den Boogaard et al.,

2000). Second premolars and third molars were the most affected teeth in this family and in another report (Vastardis, 2000).

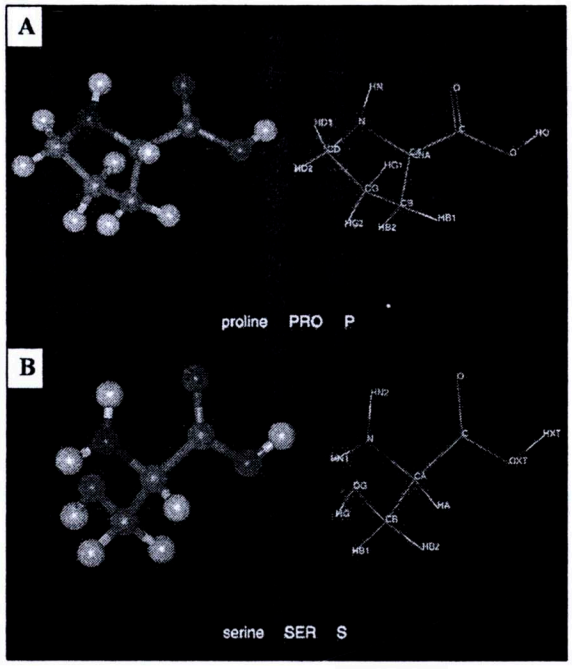

Figure 5.1 Comparison of proline and serine structure models. A) Proline is unique among the 20 standard amino acids in that the amino group is part of the cyclical ring of atoms. This unique aspect of proline is important in establishing the structure of the protein (bending). B) Serine has a short group ended with a hydroxyl group. Its hydrogen is easy to remove, so serine is very hydrophilic. The outer regions of soluble proteins tend to be rich with them (adapted from http://www.imb-jena.com).

Regarding association between missing teeth and microdontia, Lidral and Reising (Lidral and Reising, 2002) reported variations in tooth phenotypes of *MSX1* mutations. They found an *MSX1* mutation in a family with hypodontia and also found that the existing teeth were smaller, maxillary second molars lacked the disto-lingual cusp and mandibular first molars lacked the disto-buccal cusp. These findings suggest that *MSX1* may play a role in both the morphogenesis and patterning of the tooth. Some studies have considered the variations in shape and size, like peg-shaped teeth, as a variation in the expression of the mutated genes (Arte et al., 2001; Lidral

and Reising, 2002). The activity of modifying genes or epigenetic factors is regulated by the interaction with other molecules that can be tissue-specific and have allelic variants (Jumlongras et al., 2001). The interaction could result in different phenotypes. However, hypodontia in proband I and his brother might have been caused by the mutation of another gene, that might have been the cause of hypodontia of all third molars in their mother.

Of interest is the finding that the proband has limb anomalies. This is the first report that *MSX1* mutation is associated with cleft lip and palate, hypodontia and upper limb anomalies. Concerning the association between limb anomalies and *MSX1*, Hwang and colleagues have reported the association between *MSX1* polymorphism and limb deficiency defects, as well as potential interaction between maternal smoking and the infants' *MSX1* polymorphism in the risk of limb deficiency (Hwang et al., 1998). Their subjects included patients with polydactyly, with syndactyly, with upper limb anomaly, with lower limb anomaly, and with another isolated birth defect. Among these patients with limb deficiency defect, the frequency of carrying *MSX1* polymorphism was significantly higher. It is interesting to note that the proband I in this study had polydactyly similar to that found in the subjects in their study.

The role of the *Msx1* gene in limb formation has been studied in animals (Catron et al., 1996; Tabin, 1991). *Msx1* expression is located in the apical ectodermal ridge and in the subjacent distal mesoderm at an early stage of chick limb-bud development. *Msx1* has deeper expression in anterior cells compared to posterior, suggesting that *Msx1* may be involved in specification of anterior positional identity. At a later stage,

Msx1 is expressed in the zones of apoptosis or programmed cell death, indicating that *Msx1* may play a role in shaping the developing limb bud (Coelho et al., 1993).

Comparing the phenotypes between proband I in this study and the affected individuals in a previously reported mutation (p.G267C) that is located at the same C-terminal side of the homeodomain protein and adjacent to p.P241S mutation (Tongkobpetch et al., 2006), the author found that the patient with the G267C mutation had cleft lip and palate similar to that found in proband I in this study. Hypodontia and limb anomalies were not found in the patient with the p.G267C mutation. These different phenotypes might be caused by the effects of modifier genes, by the modulation of gene-environment interaction, and/or by variable expressivity.

Family II

In addition to the p.P241S mutation, this study detected a novel heterozygous missense c.589G>A (p.A197T) mutation in *MSX1* in a Thai patient affected with unilateral right cleft lip and palate and hypodontia of the right maxillary permanent lateral incisor. This mutation was also found in this patient's mother, who had microdontia of the right maxillary permanent lateral incisor, and in the patient's sister, who had hypodontia of the right mandibular permanent third molar. This novel missense mutation might be responsible for orofacial clefts and hypodontia in this family. There are several possible explanations for supporting this opinion.

First, p.A197T was not present in 200 chromosomes from the 100 control individuals of Thai ethnic background. Second, the ClustalX amino acid homology quality score revealed that this alanine is conserved in the zebrafish, African clawed

frog, chicken, house mouse, brown rat, cow, dog and chimpanzee, suggesting that this alanine has important function in living organisms. In addition, there is a substantial change in amino acid class, from non-polar alanine to polar threonine (Figure 5.2).

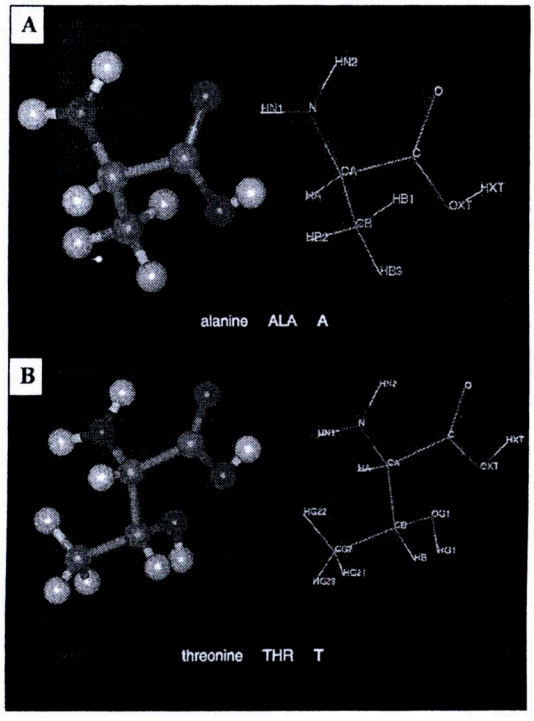

Figure 5.2 Comparison of alanine and threonine structure models. A) In alanine, the α-carbon atom is bound with a methyl group ($-CH_3$), making it one of the simplest α-amino acids with respect to molecular structure. B) Threonine has a short group ended with a hydroxyl group. Threonine is very hydrophilic, so the outer regions of soluble proteins tend to be rich with them (adapted from http://www.imb-jena.com).

This p.A197T mutation is located in helix II of the homeodomain, which is located close to the backbone of the α-strand of DNA (Figure 5.3), and may use a salt bridge to interact specifically with the G-5 phosphate group of DNA. The homeodomain, which consists of 60 highly conserved amino acids, is an important part of the MSX1 protein that is involved in protein stability, DNA binding

specificity, transcriptional repression and protein interactions (Zhang et al., 1996; Zhang et al., 1997). In addition to its interaction with DNA, the Msx1 homeodomain has physical interaction with proteins that are important for the biological process of Msx1 such as distal-less homeobox (Dlx) (Zhang et al., 1997), TATA box binding protein (TBP) (Catron et al., 1995), general transcription factor TFIIF (Zhang et al., 1996), Pax3 (Bendall et al., 1999), Pax9 (Ogawa et al., 2006), Lhx1 (Bendall et al., 1998); and Dhx2 (Bendall et al., 1998; Bendall et al., 1999). Moreover, Msx1 is also able to bind with Msx1 as homodimeric complexes or with Msx2 as heterodimeric complexes (Zhang et al., 1997). Therefore, the p.A197T mutation may result in abnormal protein structure and may interrupt these normal *MSX1* protein stability, DNA binding specificity, transcriptional repression and protein interactions. Third, protein encoded from p.A197T was predicted to be "probably damaging" by PolyPhen (http://genetics. bwh.harvard.edu/pph/).

This novel p.A197T mutation of *MSX1* is located next to the p.A194V and p.R196P mutations in previous reports (Mostowska et al., 2006; Vastardis et al., 1996). Those reports demonstrated that the p.A194V and p.R196P mutations were associated with hypodontia in humans. Functional analysis of p.R196P mutated protein by Hu and coworkers showed that the ability to transcriptional repression, DNA interaction, and interaction with other proteins were severely impaired (Hu et al., 1998). They also showed that p.R196P did not affect the activity of wild-type Msx1. They proposed that the phenotype of patients with selective tooth agenesis is due to haploinsufficiency, because of the assumption that the decreased dosage of *MSX1* in other teeth is tolerated, whereas morphogenesis of the affected teeth requires a greater dosage of *MSX1*. When comparing the phenotype between the patient in this

study with p.A197T and the patients in the previous reports, it was noted that the patient in this study had cleft lip and palate, which was not found in the patients reported with p.A194V and p.R196P mutations.

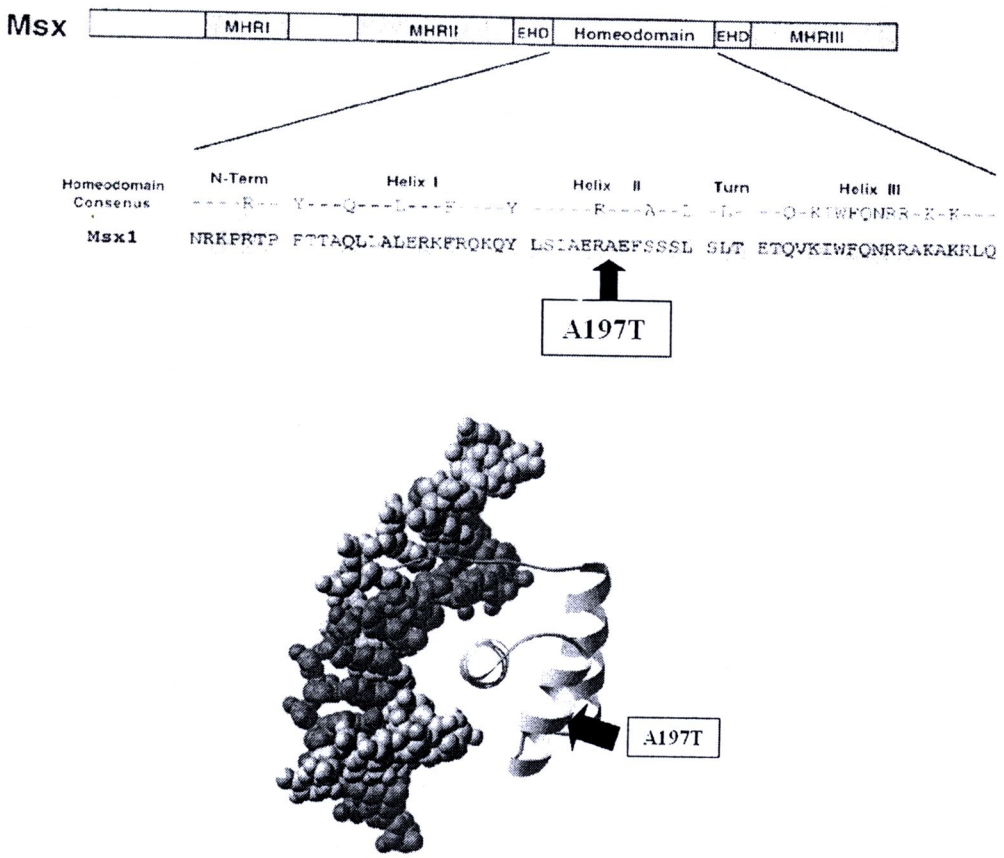

Figure 5.3 the p.A197T mutation is located in helix II of the homeodomain, which is located close to the backbone of the α-strand of DNA. The black arrows indicate the positions of p.A197T (adapted from Hovde et al., 2001).

Regarding the pattern of hypodontia, the patient with the p.A194V mutation has been reported to have hypodontia of both mandibular permanent central incisors, some permanent first premolars, all permanent second premolars, some permanent second molars, and all permanent third molars (Mostowska et al., 2006). Patients with the p.R196P mutation have been reported to have hypodontia of some maxillary

permanent first premolars, all permanent second premolars, and all permanent third molars (Vastardis et al., 1996). It is interesting to note that the most affected teeth in these two previous studies were premolars and third molars, whereas lateral incisors and third molars were the most affected teeth in the family in this study with p.A197T. A similar pattern of tooth agenesis has been reported in several studies of *MSX1* mutations (Vastardis et al., 1996; van den Boogaard et al., 2000; Jumlongras et al., 2001).. The lower second premolars were the most commonly affected, followed by upper second premolars, upper first premolars, and upper lateral incisors, respectively. Third molar agenesis, in the case of complete absence, has been suggested to have a causal relationship with the *MSX1* mutation (Vastardis et al., 1996). There may be a pattern of anterior progression of agenesis for each tooth type, because the most posterior tooth of each tooth type is most often affected, and the severity worsens with more posterior position.

Different phenotypes or variable expressivity in the same family may be caused by the effects of modifier genes, and/or by the modulation of gene-environment interaction. Mostowska and colleagues have demonstrated a novel mutation of *MSX1*; this mutation was found not only in the proband, but also in his father and mother, who did not show any phenotype (Mostowska et al., 2006). They suggested that the different phenotypes in their reported family might be the result of incomplete penetrance, mutations of several genes or mutation of another gene. However, hypodontia of the right maxillary permanent lateral incisor in proband II in this study might be the result of the abnormal tooth development that is commonly found in the cleft site. Moreover, hypodontia of the right mandibular permanent third molar in the sister of proband II might be caused by a different factor.

However, there is the possibility that p.P241 and p.A197T may not be associated with phenotypes found in the probands in this study. The method to verify that these mutations have effects on the functions of MSX1 protein is by performing a luciferase reporter assay. Luciferase commonly is used as a reporter to assess the transcriptional activity in cells that are transfected with a genetic construct containing the luciferase gene under the control of a promoter of interest.

In addition to the p.P241S and p.A197T mutations, this study has identified a heterozygous missense 440C>A (p.P147Q) variation in *MSX1* in a Thai boy affected with unilateral cleft lip and palate. This variation was also found in his unaffected mother. This heterozygous missense variation resulting in Pro147Glu substitution is located in exon 1. The p.P147Q has previously been reported to be associated with cleft lip and palate, based on the strong conservation of the amino acid, and it was not detected in over 1,600 control individuals of various ethnic backgrounds (Suzuki et al. 2004; Vieira et al. 2005). They suggested that it was a mutation in seven Vietnamese and two Philippine patients. In addition, Vieira and coworkers also found the p.P147Q variant in unaffected members, while some affected did not carry it. This observation makes its role arguable (Vieira et al. 2005).

However, Tongkobpetch and coworkers have suggested that the p.P147Q variant is not pathogenic because they found this variant in eight of 100 Thai controls. Moreover, this variant was found in three of their 100 Thai patients with cleft lip and palate, but could not be detected in their patients with cleft lip without cleft palate (Tongkobpetch et al., 2006). In this study, p.P147Q was not present in 200 chromosomes from the 100 control individuals of Thai ethnic background.

It is interesting to note the important roles of proline 147. Proline 147 is conserved in *MSX1* genes throughout vertebrate evolution. It is located in the conserved sequence of amino acids just N-terminal to the homeodomain, previously called the extended homeodomain (EHD). This EHD region consists of a PBX (pre-B-cell leukemia homeobox) binding sequence (TPWMQ), several potential phosphorylation sites, a potential nuclear localization signal, and conserved residues that mediate homo- and hetero-dimerization of Msx1 with other transcription factors (Suzuki et al., 2004). PBX is the protein that raises the DNA binding specificity for Msx1 protein (Morgan et al., 2000). Jezewski and coworkers (Jezewski et al., 2003) found the mutation in the N-terminal domain and suggested that Msx1 protein lacking the N-terminal domain was not able to upregulate cyclin D1, nor to inhibit differentiation. Early differentiation in the progress zone of the facial processes could reduce outgrowth. In addition to the important roles of proline 147, Suazo and coworkers (Suazo et al.) analyzed five SNPs using the transmission disequilibrium test and the transmission asymmetry test, to evaluate the possible parent-of-origin effects of *MSX1* in Chilean patients with non-syndromic cleft lip and palate. They found that one of the five SNPs (rs12532) showed a 2.08-fold increased risk of developing non-syndromic cleft lip and palate in the affected offspring when this SNP was inherited from the father. Thus, they suggested that *MSX1* could show parent-of-origin effects in non-syndromic cleft lip and palate. From these supportive opinions, the author suggests that the p.P147Q variant is not pathogenic, but that this variant may increase the risk of cleft lip and palate for offspring.

Regarding the hypothesis in this study, the author concludes that *MSX1* mutations (p.P241S and p.A197T) were detected in a Thai patient with syndromic

cleft lip and palate. To verify these mutations, functional analysis of p.P241S and p.A197T, and further studies, which should have an increase in the sample size and which consider the significance of ethnic group, may improve knowledge in this field.