

CHAPTER V

DISCUSSION

All subjects were Thai individuals, who were clinically diagnosed with either HED or non-syndromic hypodontia. Here the author reported four different missense variants of the *EDA* gene: two of them caused HED, one caused non-syndromic hypodontia and the other was a single nucleotide polymorphism. All of these variants have been previously identified. Additionally, two deletions in the *EDA* gene were found. Of these one was a novel mutation located in exon 5 and the other was a known polymorphism located in an intronic region. All variants found in this study were located in exons 3, 5 or 9. This agrees with results from many studies that the *EDA* gene was highly mutated in exons 3, 5, and 9 (Monreal et al., 1998; Pakkonen et al., 2001; Schneider et al., 2001; Vincent et al., 2001). Previously published reports found that mutations within exon 5 were often deletions (Schneider et al., 2001; Vincent et al., 2001). This finding is also similar to that of the present study where the mutations found within exon 5 were deletions.

As previously described, mutations of signaling molecules in the EDA-EDAR-NF- κ B pathway can cause HED with various clinical phenotypes, and different modes of transmission, and thus can produce different disorders. *EDA* is known as the causative gene of XLHED, whereas EDAR and EDARADD causes the autosomal pattern of HED and NEMO causes incontinentia pigmenti and osteopetrosis with or without lymphoedema with HED-ID when mutated. Despite the clinical features of the X-linked and the autosomal HED being indistinguishable, their

pedigrees can help to classify them. Because the family histories of neither sporadic patient with HED (CGL DNA No.738 nor 813) show any affected individuals, molecular genetic analysis was performed to find out the causes of HED in the patients. The author chose to screen mutations in the *EDA* gene rather than in the other genes that cause HED for many reasons. First, the sporadic cases were male and their clinical phenotypes were the apparent features of hypohidrosis, hypotrichosis and hypodontia and were similar to patients seen in previous reports of *EDA*-associated XLHED. Second, the mother (CGL DNA No.740) of proband No.738 was healthy without any signs suggesting HED characteristics, whereas the mother (CGL DNA No.807) of proband No. 813 had a normal appearance, except for two peg-shaped lateral incisors. There are many reports that female carriers of XLHED can manifest a wide range of severity from normal to the complete HED features. Most affected females with ARHED (Chassaing et al., 2010; van der Hout AH et al., 2008) and ADHED (Chassaing et al., 2006) often manifest at least one phenotype, whereas female carriers of XLHED tend not to express any signs in many times (Vincent et al., 2001). In addition, these families had no history of consanguinity. Therefore, the possibility of being an autosomal-dominant or -recessive inheritance is less likely than being X-linked pattern. Finally, among genes associated with HED, *EDA* has the highest frequency of mutation reports. For all of these reasons, the sporadic cases in the present study were assumed to be affected with the X-linked HED or to carry *EDA* mutations. Satisfyingly, molecular testing to identifying causative mutations, p.Arg156Cys and p.Ala349Thr, was successful in both sporadic cases.

The p.Arg156Cys (c.466C>T) mutation was investigated in the six-year-old Thai boy affected with HED (CGL DNA No. 738). He was diagnosed with HED by the distinctive face of this syndrome and the three obvious main phenotypes of HED, hypohidrosis, hypotrichosis and hypodontia. There was a negative history of HED in this family. Molecular testing of the *EDA* gene indicated that his mother harbored that particular mutation in a heterozygous pattern. Interestingly, she had no any signs of ectodermal defects, which imply a carrier of XLHED. However, there are many reports which support the idea that healthy females might be heterozygous carriers of XLHED (Vincent et al., 2001; Huang et al., 2006).

The p.Arg156Cys mutation has been identified as being associated with XLHED in 11 unrelated patients with XLHED in six reports (Bayes et al., 1998; Monreal et al., 1998; Schneider et al., 2001; Vincent et al., 2001; Hashiguchi et al., 2003; Lexner et al., 2008). This mutation has occurred in either heritable or spontaneous conditions. Phenotypes of previously reported males with XLHED were the three classic features of hypohidrotic ectodermal dysplasia, hypohidrosis, hypotrichosis and hypodontia. Phenotypes of heterozygous carriers of p.Arg156Cys were clinically described in some reports. Missing teeth were the most common characteristic found in carriers. A distinctive face, abnormal scalp hair and a subnormal salivary secretion rate were also observed. Besides p.Arg156Cys, this proband also had IVS5+11_12delCT, which has not been found in the previously reported patients harboring p.Arg156Cys. Moreover, there has not been a report of any female carriers harboring p.Arg156Cys who were healthy without any signs of ectodermal defects, as seen in the mother carrier in the present study (Bayes et al.,

1998; Monreal et al., 1998; Schneider et al., 2001; Vincent et al., 2001; Hashiguchi et al., 2003; Lexner et al., 2008).

The significance of arginine position 156 is supported by many findings. First, EDA contains two overlapping consensus furin cleavage sites in the extracellular domain, which are constituted in the sequence of Arg¹⁵³-Val-Arg-ARG¹⁵⁶-ASN-LYS-ARG ARG¹⁵⁹ (upstream, underscore; downstream, capital letter) (Chen et al., 2001; Schneider et al., 2001). The furin recognition sites are required for the proteolytic process, which leads to the secretion of a functional TNF-containing fragment of the EDA protein. The arginine position 156 is an overlapping amino acid and shares both cleavage sites. It is now apparent that the arginine position 156 is a functionally important site for the proteolytic process of both upstream and downstream furin cleavage sites of EDA. Mutations at this position are predicted to cause a complete failure of proteolysis for paracrine signaling of the *EDA* gene.

Second, mutations within the cleavage sites account for 20-30% of all patients with XLHED (Schneider et al., 2001; Vincent et al., 2001). Of these, mutations at Arg156, affecting both consecutive consensus sites, are the most frequently identified within the cleavage sites and within the whole gene. The Arg156 position is highly mutable due to its codon of CGC, which produces C-to-T transition at CpG dinucleotides. Therefore, the arginine substitution with cysteine had the highest frequency. In addition to cysteine, this hotspot position was also replaced by histidine (c.467G>A) and serine (c.466C>A), with lower frequency (Aoki et al., 2000; Paakkonen et al., 2001; Vincent et al., 2001; Zhao et al., 2008). Third, the substitution of arginine with cysteine changes the polarity of the amino acid from basic to uncharged. Fourth, multiple alignments of amino acid sequences demonstrate the complete

conservation of the arginine position 156 among various species: zebrafish, stickleback fish, chick, cow, brown rat, house mouse, monkey, and chimpanzee. Finally, to date, the p.Arg156Cys mutation has been associated with only XLHED; the p.Arg156Cys mutation non-syndromic hypodontia.

Identification of p.Ala349Thr was carried out in the two-year-old Thai boy (CGL DNA No. 813) presenting with the distinctive facial appearance, hypotrichosis, and hypohidrosis. He was the first and only affected subject in his non-consanguineous family. Due to the high possibility of detection of *EDA* mutation, the author decided to screen mutations in the *EDA* gene first, although there was some risk of undetectable mutations in this isolated patient. As expected, DNA sequencing revealed a G to A transition at nucleotide 1045. This identified mutation has been previously reported in families affected with XLHED (Monreal et al., 1998; Kobiela et al., 2001; Paakkonen et al., 2001; Vincent et al., 2001; Na et al., 2004; Lexner et al., 2008; Shimomura et al., 2009; Zhang et al., 2011). In order to confirm the familial status, the mother of proband No. 813 was sequenced. Molecular testing revealed the same mutation in the mother.

The alanine position 349 encoded for the functional TNF homology domain of the extracellular domain, normally has an ability to interact with EDAR. The significant role of this amino acid has been attributed to many reasons. First, this amino acid residue is completely conserved among various species: zebrafish, stickleback fish, chick, cow, brown rat, house mouse, monkey, and chimpanzee. The high conservation implies the importance of the alanine position 349 for living organisms during evolution. Additionally, the substitution of alanine with threonine changes the polarity of the amino acid from non-polar to uncharged. The *EDA*

protein function may be impaired and thus result in abnormal development of ectodermal tissues. Second, this particular mutation commonly occurs and is identified in various ethnic populations (Monreal et al., 1998; Lexner et al., 2008; Shimomura et al., 2009; Zhang et al., 2011). Third, clinical findings of all males affected with the p.Ala349Thr mutation, including the current proband, are severe HED. No patients affected with this mutation present with isolated hypodontia. Although the pathogenic difference between mutations of the *EDA* gene causing XLHED and non-syndromic hypodontia is still unclear, the p.Ala349Thr variant seems to be a significantly causative mutation, accounting for only XLHED cases. Fourth, this variant was not found in 100 unrelated healthy Thai controls (150 X-chromosomes) in this study, suggesting that this variant is likely to be a pathogenic mutation. This study further confirmed that mutations in the *EDA* gene can cause XLHED syndrome.

There is a wide variability of phenotypes in female carriers. Their phenotypes range from no manifestations to the complete phenotype of HED. The reason for variable degrees of phenotypes in female carriers is the process of random inactivation of one of the two X chromosomes (lyonization). This process results in a mosaic pattern of two different progenitor cells: active and inactive cells. The severity of carrier expression varies considerably on the ratio of these cells. Many clinical features have been observed in heterozygous carriers of XLHED. Many reported studies have differently focused their attentions on the clinical abnormalities of heterozygous carriers (Cambiaghi et al., 2000; Lexner et al., 2008). Most female carriers have mild clinical manifestations (Vincent et al., 2001; Lexner et al., 2008). Dental abnormalities are the most common. Mild alterations in skin, scalp and body

hair hypotrichosis, and some facial features are also frequently described. Additionally, female carriers present with mosaic distribution along Blaschko lines on their backs, as demonstrated by the starch-iodine test (Cambiaghi et al., 2000). Although a careful physical examination is necessary and is a way to help in diagnosing carriers, it is difficult to establish the diagnosis in suspected female carriers of XLHED and to differentiate a female carrier of XLHED from a woman affected with ARHED, especially if she is asymptomatic. The mutation analysis of the first two families underlined that the heterozygous carriers associated with *EDA* mutations can appear normal without any of the three signs of hypotrichosis, hypohidrosis, or hypodontia. Because the mother of one proband had microdontia of the upper lateral incisors, this study suggests that microdontia may be an additional sign of an XLHED carrier.

Another mutation found in this study was p.Glu164Ala, which was detected in two related cases whose clinical phenotypes were isolated hypodontia of permanent teeth. The proband (CGL DNA No. 431) had hypodontia of two maxillary canines and two maxillary third molars. Her father (CGL DNA No.811) had hypodontia of two maxillary third molars. Mutation analysis of the *EDA* gene from this family showed a single nucleotide substitution (c.491A>C) in exon 3, replacing the highly conserved glutamic acid position 164 by alanine. This substitution of amino acid altered the polarity from the acidic state to non-polarity. The amino acid position 164 resides between the furin recognition site and the collagen-like domain. Its role has not yet been discovered. However, based on its conservation and its alteration of polarity, this variant is predicted to be a causative mutation.

In contrast, a previous study found this mutation in a Chinese family with XLHED (Fan et al., 2008). In that report, although the family included a male with XLHED, the only family member described clinically and genetically was a heterozygous female carrier. In addition to absent teeth, other ectodermal abnormalities were also observed in that carrier. Her phenotype included hypoplastic skin, wrinkling, sparse hair, typical facial features and severe tooth loss. She had only five teeth (teeth 16, 21, 26, 36, and 46). However, there was no detailed mutation analysis of the patient male with XLHED. The unclear data in that report raises the question of whether p.Glu164Ala, which was detected in the carrier of that report and in proband 431 of this present study, is a potential mutation. Screening this variant in 100 normal controls was performed in this study to elucidate that question.

Eda activity has crucial roles in tooth morphogenesis. In mice, in the absence of *Eda*, tooth buds are small and dental organs seem to be more hypoplastic, resulting in small teeth with few cusps in adults (Mikkola and Thesleff, 2003). Overexpression of *Eda* leads to supernumerary teeth distal to the third molars in mice (Tucker et al., 2004). Studies of tooth agenesis-causing mutations have demonstrated that anterior teeth require the highest level of EDA signaling and posterior teeth appear to have less requirements and form normally in response to *EDA* mutations (Mues et al., 2010). Tooth agenesis-causing *EDA* mutations have been shown to cause impairment of the EDA expression, the receptor binding and the signaling ability of the mutant protein. However, the XLHED-causing *EDA* mutations have been demonstrated to abolish EDA expression, receptor binding and signaling (Mues et al., 2010). This finding emphasizes the crucial roles of EDA in tooth formation.

In this study, the analysis revealed a single new mutation, p.Glu164Ala, producing different phenotypes of XLHED and non-syndromic hypodontia. With the exception of the p.Glu164Ala mutation, there are only two identified mutations associated with both XLHED and isolated hypodontia (Schneider et al., 2001; Lexner et al., 2008; Li et al., 2008). Both mutations are missense mutations and are located in only exon 9. The first, p.Ser374Arg, was identified in an affected male with only isolated hypodontia, in a family whose other members exhibited complete phenotype of XLHED (Schneider et al., 2001). This missense mutation demonstrated weak but specific binding to Edar *in vitro*. This suggests intra-familial variability. The second mutation causing both disorders is p.Asp316Gly, previously reported in three unrelated families (Lexner et al., 2008; Li et al., 2008; Zhang et al., 2011). One of the three families affected with the p.Asp316Gly mutation had isolated hypodontia (Li et al., 2008). The amount of tooth loss in those families was variable, ranging from eleven missing teeth to anodontia. In contrast, the other two families are affected with XLHED and presented with anodontia, hypotrichosis, hypohidrosis, and HED facial features (Lexner et al., 2008; Zhang et al., 2011). Other members in those families also harbored the p.Asp316Gly mutation.

This is the first report identifying the p.Glu164Ala mutation in exon 3 producing two different *EDA*-associated disorders, rather than in exon 9. However, the pattern of mutation is still missense. This suggests the hypothesis that tooth agenesis is a variable expression of the XLHED syndrome. The severity of the dental phenotype appears to correlate with the general clinical picture of the patients with XLHED (Clauss et al., 2010, Zhang et al., 2011). Missense mutations in *EDA* that cause isolated hypodontia have been suggested as being either hypomorphic

(Schneider et al., 2001) or minimally affecting the stability of EDA trimers (Li et al., 2008). Another explanation of the same mutation causing different clinical features is probably that dental tissue, which is affected in both disorders, may be more sensitive to the mutation than are other ectodermal tissues and normally needs the highest levels of EDA-associated interaction during development (Mue et al., 2010). A compensatory pathway may be absent in families with a more severe phenotype (XLHED). Moreover, other evidence has raised the alternative explanation that the different phenotypic expression is dependent upon other factors, including modifier genes, environmental factors, and epigenetic factors. To date, only 11 mutations have been found in *EDA*-associated non-syndromic hypodontia (Tao et al., 2006; Tarpay et al., 2007; Song et al., 2009). Presumably, the wide range of phenotypic severity resulting from the same *EDA* mutations could be more obvious if mutations causing isolated cases were more frequently detected. However, there are no clearly published data to elucidate the molecular relationship between and pathogenesis of XLHED and non-syndromic hypodontia.

In addition to p.Glu164Ala, this study also identified the c.1001G>A transition, resulting in a substitution of p.Arg334His in the family V affected with non-syndromic hypodontia. The proband (CGL DNA No. 728) had hypodontia of six teeth, 13, 15, 23, 25, 33, and 35. His father (CGL DNANo. 810) had only hypodontia of tooth 12 (right maxillary lateral incisor). His mother had slightly sparse hair and a complete permanent dentition. No other abnormalities were found in members of this family. The average percentage of hypodontia for upper lateral incisors is approximately 75%, (Zhang et al., 2011), almost as high as the highest percentage among all tooth types (77% for the lower lateral incisors). This p.Arg334His

mutation was reported in a Chinese man, 18 of whose permanent teeth were absent (Figure 5.1). His congenitally missing teeth consisted of all maxillary and mandibular permanent lateral incisors, canines, and premolars and mandibular permanent central incisors (Song et al., 2009). The number of missing teeth of the patient in that report was higher than that in this report. Regarding the position of missing teeth in non-syndromic hypodontia, *EDA* mutations seem to affect mainly lower incisors and upper lateral incisors (Han et al., 2008; Zhang et al., 2011). The pattern of hypodontia could not be summarized, due to number of cases (only two related patients affected with the p.Glu164Ala mutation) in this study.

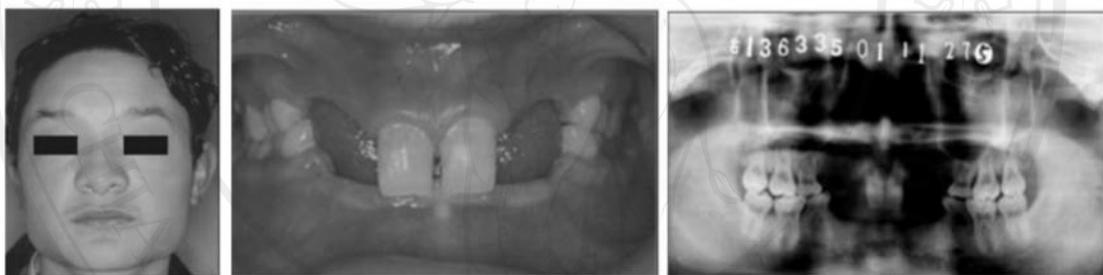


Figure 5.1 Phenotype of a Chinese man affected with p.Ar334His. Panoramic radiograph shows multiple absent teeth in this patient but his face and otherwise features are normal (Modified from Song et al., 2009).

The Arg334 position is located in the C-terminal TNF homology domain of the *EDA* protein. The Arg334 position is highly conserved during evolution of various species. Structural analysis of the *EDA* protein has demonstrated that three Arg334 residues construct a hydrogen-bond-interacting network in the *EDA* trimers (Song et al., 2009) (Figure 5.2).

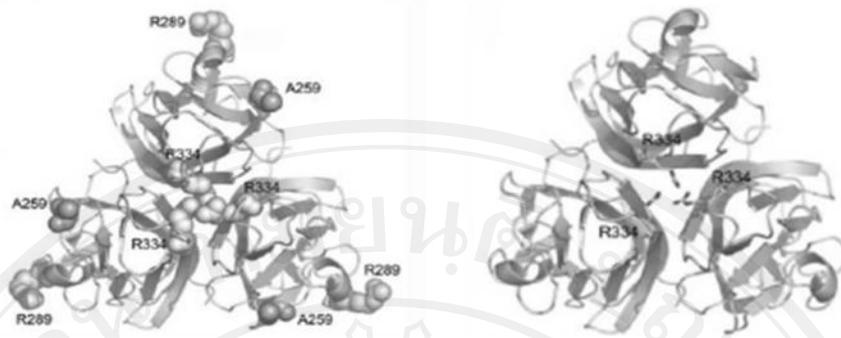


Figure 5.2 Structural analysis of the Arg334. EDA protein has three Arg334 residues which interact with each other, leading to construction of a hydrogen bond (Modified from Song et al., 2009)

Another family, a five-generation Thai family affected with XLHED, harbored the same variant (p.Arg334His) in this study. All affected males exhibited the complete manifestations of HED and their pedigree showed the X-linked form of inheritance. The family members all had maxillary permanent central incisors which appeared cone-shaped, and most of the affected males (three out of the four) had at least one maxillary first molar. In accordance with published data, the teeth most often present in patients with XLHED were the maxillary permanent first molar followed by maxillary permanent central incisor (Lexner et al., 2007; Zhang et al., 2011). Although most of the maxillary permanent central incisors remained, they often became conical. The phenotypes of affected individuals in this family were similar to those of previous reports.

In the large five-generation family with XLHED in this study, DNA sequencing revealed c.646-663del18 within exon 5, in addition to the p.Arg334His variant in exon 9. The 18-bp in-frame deletion led to a lack of six amino acids which eliminated two Gly-X-Y repeats in the collagen domain in the extracellular domain of *EDA*. Four subjects affected with XLHED and two female carriers harbored this 18-

bp deletion. In order to exclude this variant from the category of polymorphism, one hundred ethnically matched normal controls were also sequenced. None of the normal controls carried this deletion, suggesting that c.646-663del18 was a novel mutation in *EDA*.

The overall conservation between *EDA* and *Ta* protein is high (94%). However, from the beginning position (amino acid sequence 180) of the Gly-X-Y collagen domain through the ending position (amino acid sequence 238), all *EDA* sequences are completely identical to those of *Ta* (Bayes et al., 1998). This high conservation particularly supports the importance of the sequences in the Gly-X-Y collagen domain to the process of evolution. Evidence has shown that the collagen domain plays a role in the multimerization of *EDA* trimers (Ezer et al., 1999; Schneider et al., 2001). However, it is not clear whether the collagen domain of *EDA* has other functions, since the effects of *EDA* mutations within the collagen domain are different, depending on the types of mutation, under *in vivo* conditions. The cytotoxic activity of recombinant proteins has been investigated and reflected the ability of multimerization of the *EDA* proteins. Interestingly, a previous study revealed that in-frame deletions of 2 or 4 Gly-X-Y repeats do not prevent multimerization of the TNF homology domain, but point mutations within the same region completely prevent the multimerization. There is no clear explanation of effects of in-framed deletions in the collagen domain. Some investigators have suggested that the collagen domain of *EDA* may have other functions, such as those of other ligands containing a collagen domain, e.g., interaction with other molecules (Ezer et al., 1999; Schneider et al., 2001). Nevertheless, other functions of the collagen domain is unknown, the in-frame deletions in that region shortening Gly-X-

Y repeats resulted in three-dimension structural protein changes which might predicted to effect the protein function. However, in-frame deletions have frequently occurred and have been found in many XLHED patients (Schneider et al., 2001; Vincent et al., 2001; Lexner et al., 2008). The biological importance of the collagen domain of EDA needs further clarification. The c.648-665del18 mutation, which resulted in p.Pro216fs, is the nearest mutation to c.646-663del18 (Zhang et al., 2011). The patient affected from the c.648-665del18 was three typical signs of XLHED. Mental development was normal. He had a characteristic facial appearance, including a prominent forehead, saddle nose, and protruding lips.

The results of this study reveal that both different variants in family II with XLHED, c.646-663Del18 and p.Arg334His, concurred in individuals with XLHED and in heterozygous carries of this family. Correspondingly, one unaffected male (CGL DNA No. 816) in the family (although his mother was a carrier) and his younger brother (who was affected with XLHED), did not carry either mutation. Moreover, p.Arg334His was also detected in another family (family V) affected with non-syndromic hypodontia, leading to the question whether the p.Arg334His really is a pathogenic mutation as a previous report concluded, or only a nonpathogenic polymorphism. The finding in this study supports the idea that c.646-663Del18 is a pathogenic mutation causing XLHED. Contrastingly, screening for this variant in 100 unrelated normal individuals detected the p.Arg334His variant in three females of them. Therefore, the possibility of p.Arg334His carrier in the three female controls is remained. In order to differentiate p.Arg334His variant between a single nucleotide polymorphism and a pathogenic mutation, other methods are required. The easiest way to classify is finding noemal males who are hemizygous p.Arg334His variant

which would indicate polymorphism. Others method such as statistical methodology (Fisher's exact test) and functional analysis should be performed unless there is indication of polymorphism from the first suggested method.

In this study, *EDA* mutations were mainly detected in (Families I, II, and III) with XLHED rather than in the families (Family IV) with non-syndromic hypodontia. To date, *PAX9* (Klein et al., 2005; Kapadia et al., 2006), *MSXI* (van den Boogaard et al., 2000; Mostowska et al., 2006), *AXIN2* (Callahan et al., 2009), *WNT10A* (Kantaputra and Sripathomsawat, 2010) and *EDA* mutations have been identified as the etiologies of non-syndromic hypodontia. In this study, mutations have remained undetected in several isolated cases of hypodontia from direct sequencing in *EDA*. This suggests that they may result from mutations of other known causative genes or other undiscovered genes.

Mutation detection rates vary from 63% to 95% (Monreal et al., 1998; Schneider et al., 2001; Vincent et al., 2001), depending on case selection and detection technique. Direct sequencing results in the highest detection rate of mutations. However, in this study, there was one patient (CGL DNA No. 787, Figure 5.3) affected with XLHED whose mutation has not yet been detected. The disruption of a gene may be caused by abnormalities in any parts of the gene, but such disruptions are not detectable from DNA sequencing. In order to identify those disruptions, RNA analysis is required.

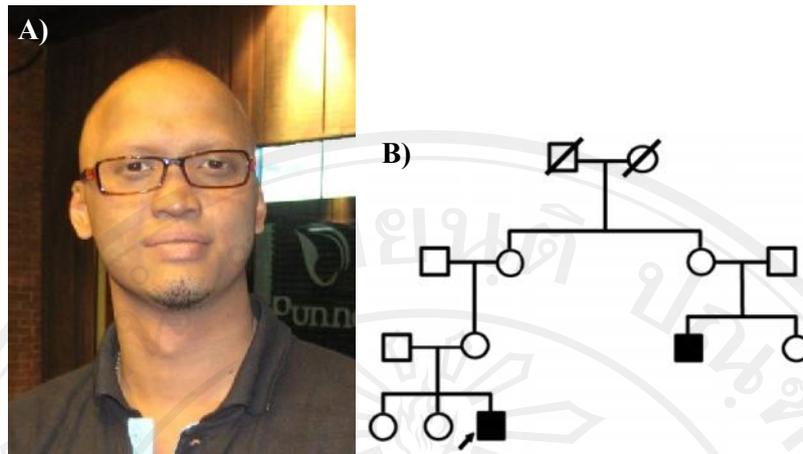


Figure 5.3 Proband CGL DNA NO.787 whose mutation has not been found. A, Proband had typical phenotypes of HED B, Pedigree of his family shows clear X-linked inheritance.