CHAPTER II

REVIEW OF THE LITERATURE

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2.1 *EDA* gene

2.1.1 *EDA* structure and EDA protein

The human *EDA* (*ED1*) gene is located in the X chromosome, causing hypohidrotic (or anhidrotic) ectodermal dysplasia in males and isolated hypodontia when mutated (Kere et al., 1996; Kobielak et al., 2001; Paakkonen et al., 2001; Schneider et al., 2001; Vincent et al., 2001; Tao et al., 2006; Tarpey et al., 2007). The *EDA* gene maps to Xq12-q13.1 and contains twelve exons (Bayes et al., 1998).

The *EDA* gene was undergone several alternative splicing either from human or mice tissue, producing several isoforms (Kere et al., 1996; Bayes et al., 1998; Monreal et al., 1998). In 1996, the *EDA* gene was first isolated by positional cloning, and coded for a truncated incomplete molecule (Kere et al., 1996). *EDA-O* which is the first and shortest transcript encodes 135 amino acids containing collagen-like domains in the extracellular domain, but lacking a TNF-binding domain. This transcript is composed of 858-bp cDNA and consists of two exons (exon1 and exon2), separated by a 200 kb intron. Multiple alternative splicing of the nine coding exons of *EDA*, has been created eight different isoforms of its transcripts. All of them are transmembrane proteins, share exon 1 and encode a short intracellular domain and an extracellular domain (Kere et al., 1996; Bayes et al., 1998; Monreal et al., 1998). The *EDA* gene has amino acid conservation in all vertebrates. The overall human homology to the Tabby (Ta) murine protein is approximate 95% and within the TNF binding domain is 100% identity (Bayes et al., 1998).

Among all various transcripts of EDA, only two isoforms of EDA (EDA-A1 and EDA-A2) have clearly been shown to have biological activity *in vitro* (Yan et al., 2000). Both closely related isoforms contain eight exons (exons 1 to 9, except exon

2, which appears only in *EDA-O*) (Table 1) and encode for proteins containing 391 and 389 amino acids, respectively (Bayes et al., 1998). Because of the alternative usage of splice donor sites within IVS8, EDA-A1 has two more amino acids (Val307 and Glu308) in the TNF-binding domain than does EDA-A2, leading to different isoforms and to alternative binding domains. The 1.4-kb human cDNA of *EDA-A1* contains about 600 bp that are identical to those in exon 1 of other transcripts, as previously described (Kere et al., 1996), followed by a 780-bp unique DNA sequence. The genomic structure of the EDA-A1 gene has been established, and the complete sequence of seven additional exons has been determined in human XLHED-affected males (Monreal et al., 1998) (Table 1). Detection rates of putative mutations within the EDA-A1 gene in patients affected with XLHED have measured at up to 95% (Monreal et al., 1998), whereas screening in the *EDA-O* produced a rate of only 7%.

Table 2.1. Exons of human *EDA-A1* gene and their exon size

Exon	Exon size (bp)	cDNA (nt)
1	638	1-638
3	106	639-744
4	24	745-768
5	180	769-948
6	35	949-983
7	52	984-1035
8	131	1036-1166
9	252	1167-1418

The two longest isoforms, EDA-A1 and EDA–A2, are type II transmembrane proteins with an extracellular portion containing a short collagenous domain, a TNF homology domain and a furin cleavage site (Figures 2.1). EDA is unique among the

members of the TNF ligand family, possessing three collagenous repeat domains, whereas others do not (Ezer et al., 1999; Mikkola et al., 1999). Like other ligands of the TNF family, EDA is displayed in a trimeric form. It is conceivable that the collagenous and the TNF domains are needed for their activity, including multimerization, contact and stability. The protease recognition site (most likely for furin protease) is formed by CpG rich sequences which codes for two overlapping cleavage sites, thus results in the highest mutation. Each isoform of EDA is truncated at the furin recognition site and can be released from cell membrane, suggesting that each can act as a secreted ligand and remain as a homotrimeric TNF structure (Elomaa et al., 2001). The secreted trimer EDA has been confirmed to transport and bind to the extracellular trimer domain of its receptor, in the manner of each ligand's monomer to each receptor's monomer interface, by *in situ hybridization*. Thus, EDA mediates the signal necessary for the ectodermal development. The different binding domains of EDA-A1 and EDA-A2 result in receptor binding specificity. EDA -A1 with the EDAR protein acts as a ligand-receptor pair, whereas the EDA-A2 ligand specifically matches to XEDAR (Yan et al., 2000; Elomaa et al., 2001). Conclusively, each part of the EDA structure has its specificity and necessity: the TNF homology domain necessary for receptor binding, the bundle-forming collagen domain necessary for multimerization, and the cleavage site for a furin protease necessary for proteolytic processing, which alters EDA to the secreted form. The effect of EDA can be expected to be mediated by NF-κB signaling via its transcriptional targets.

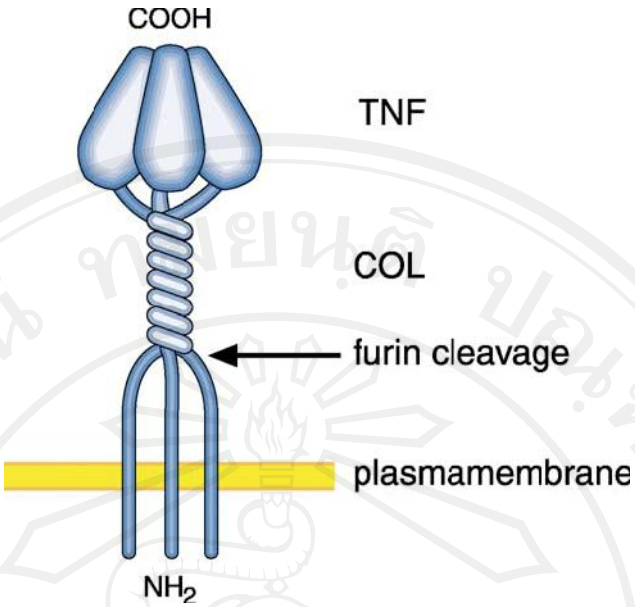

Figure 2.1 Schematic representation of ectodysplasin. Ectodysplasin is a transmembrane protein, composing of the furin recognition site, where the proteolytic process occurs for cleaving EDA to a soluble form, the collagen-like repeat (COL), necessary for multimerization, and the TNF homology domain, responsible for receptor binding specificity (Modified from Mikkola and Thesleff; 2003).

2.1.2 EDA receptors and adaptor

(1) Ectodysplasin A-1 receptor (EDAR)

EDAR, a member of the tumor necrosis factor receptor (TNFR) superfamily, is a type I transmembrane protein with a single transmembrane domain (Headon and Overbeek, 1999; Monreal et al., 1999). The *EDAR* gene maps to 2q11-q13, contains twelve exons and codes for a 488-amino-acid protein which is divided into three parts: an extracellular domain containing cystein-rich repeats domain, a single transmembrane region and a C-terminal intracellular domain. The murine *downless (dl)* gene has been positionally cloned and found to code for the ectodysplasin receptor (Headon and Overbeek. 1999). The *EDAR* gene causes either autosomal recessive (AR) or autosomal dominant (AD) HED, when mutated either in mice or

humans (Headon and Overbeek. 1999). Due to phenotypic similarities between *Tabby* (the homologue of the human *EDA* gene) and *downless* (the homologue of the human *EDAR* gene*)* mice (Monreal et al., 1999), it is likely that Edar is the receptor of the Eda (Headon and Overbeek. 1999). Later results by several groups have corroborated that Eda-A1 certainly interacts with Edar (Tucker et al., 2000; Yan et al., 2000; Elomaa et al., 2001; Kumar et al 2001). The extracellular domain of EDAR, responsible for ligand binding, is formed to trimerize. Then, EDAR exclusively binds to the TNF domain of its ligand before the transduction of signals to downstream molecules (Yan et al., 2000).

Most members of TNF receptor family lack an intracellular death domain and thus directly recruit members of the TNF-receptor-associated factor (Traf) family to mediate signaling. Contrastingly, EDAR possesses the intracellular death domain, it suitably interacts with TRAF family members and it requires specific death domain adaptors for mediating the NF-κB pathway (Hofmann, 1999). The intracellular portion of EDAR acts as a scaffold for other downstream molecules. Although the co-immunoprecipitation assay has shown that EDAR interacts with various TRAF family members and with NIK in the 293T cell line, it has failed to detect the physical interaction between these proteins. Possibly, the interaction between EDAR and TRAFs need other molecules acting as intermediate bridging proteins. The search for the respective adaptor molecule has been successful; the death domain adaptor for EDAR signaling has been identified as EDARADD (Kumar et al., 2001; Yan et al., 2002).

Mutations in *EDAR* cause autosomal ectodermal dysplasia, either recessive or dominant forms. Pattern of inheritance depends on the effect of the mutation on the

functional structure of EDAR. The extracellular ligand-binding domain of EDAR is responsible for ARHED (Monreal et al., 1999; Chassaing et al., 2006); a mutation in the intracellular domain causes either the autosomal recessive or dominant form (Chassaing et al., 2006). The autosomal forms of HED are less common than the X-linked form (XLHED), and to date, about 20 mutations in *EDAR* have been found in patients with HED. The clinical features, such as sparse hair, missing teeth, and reduced sweating ability, of the autosomal forms of HED resulting from EDAR mutations are indistinguishable from the symptoms found in the X-linked form (Munoz et al., 1997).

(2) XEDAR

XEDAR (X-linked ectodysplasin receptor or EDA2R), another member of the TNF receptor superfamily, plays the same in NF-κB activation as does EDAR (Yan et al., 2000). XEDAR-hFc in immunoprecipitation has been designed to test which isoform of EDA can bind to XEDAR. Interestingly, that procedure has shown that XEDAR exclusively interacts with EDA-A2. Essentially, there is no cross-interaction between the EDA-A1 and EDA-A2 isoforms of EDA, despite there being a difference between them of only two in the number of amino acids.

The *XEDAR* gene is located on the X chromosome and encodes two alternatively-spliced isoforms (297-amino-acid and 318-amino-acid proteins), which differ in the cytoplasmic domain (Yan et al., 2000). XEDAR, lacking a death domain, has 40 fewer amino acids than does EDAR. XEDAR, type III transmembrane protein, composes an extracellular region containing three cysteine-rich domains and a single transmembrane region, and an intracellular C-terminus without an N-terminus. Both isoforms of XEDAR can activate the NF-κB pathway but they require

some additional binding molecule to mediate further signal transduction. The cytoplasmic region is able to bind directly to TRAF1, -3, and -6. Among the TRAF molecule, TRAF6 is likely a key adaptor molecule in the XEDAR-NF-κB pathway because XEDAR possesses the specific TRAF6 binding site. This indicates that XEDAR is correlated with activation of NF-κB.

Xedar is expressed in the hair bulb and hair matrix of adult mice (Botchkarev et al., 2005). Mutation in XEDAR has not yet caused any human disorder. Xedar knock-out mice have not also been demonstrated any significant phenotype (Brosh et al., 2010). However, many single-nucleotide polymorphisms have been reported to be associated with androgenetic alopecia, a heritable disorder which is characterized by hair loss (Brockschmidt et al., 2008; Prodi et al., 2008). Moreover, this gene has been identified as p53 potential target gene (Tanikawa et al., 2009) and implicates in p53-dependent apoptosis of hair follicles in both mice and humans (Brosh et al., 2010).

(3) EDARADD

EDARADD (Ectodysplasin receptor-associated death domain, OMIM 606603) maps to chromosome 1q42.2-q43 in human (Perou et al., 1997) and chromosome 13 in mouse. Mutations in *EDARADD* cause autosomal recessive HED, both in humans and mice. The human *EDARADD* gene is composed of six exons of 208 amino acids (Headon et al., 2001). EDARADD is a cytoplasmic adaptor protein and links the intracellular death domain of EDAR to NF-κB downstream signaling molecules. Its structure contains an N-terminal Traf-binding consensus sequence (Pro-Ile-Gln-Asp-Thr) and a C-terminal death domain. There is 96% conserved identity in the death domain between human and mouse, and 80% in the overall structure. The

cytoplasmic death domain of EDAR is necessary for binding to the death domain of EDARADD for receptor engagement (Headon et al., 2001; Yan et al., 2002). *In situ* hybridization has shown that Edar and Edaradd are co-expressed in developing epithelium during hair follicle formation and in enamel knot during tooth morphogenesis in wild type mouse (Headon et al., 2001). Mutation with the intentionally truncated cytoplasmic domain of Edar abolishes Edaradd binding ability. Edaradd has been found to associate with Traf1, -2 and -3, but not with Traf4 (Headon et al., 2001; Yan et al., 2002). *In vitro* study has shown that the deletion within the Traf-binding domain, (EdaraddΔ34-40), which normally required for signal transduction, decreases the binding ability of EDARADD to EDAR, and results in loss of NF-κB signaling.

Crinkled (cr; the homologue of the human *EDARADD* gene) mutant mice have an autosomal recessive hypohidrotic ectodermal dysplasia phenotype, identical to that seen in both Tabby and Downless. Previous reports have identified a lower frequency of human mutations in *EDARADD* than in EDA or in EDAR (Headon et al., 2001; Bal et al., 2007; Chassaing et al., 2010). To date there are three reports of EDARADD mutation, two of which identified in families with ARHED (Headon et al.,2001; Chassaing et al.,2010) and the other in which of ADHED (Bal et al.,2007).

2.1.3 EDA-EDAR-NF-κB signal transduction pathway

There is evidence that stimulation of the ectodysplasin pathway by ligand binding to its receptor, EDAR, triggers binding of the intracellular EDARADD, resulting in activation of the NF-κB (Yan et al., 2000; Kumar et al., 2001) (Figure 2.2). NF-κB, previously identified as the transcriptional regulator, is crucial in transducing EDA signaling. EDA regulates the development of ectodermal

appendages in multiple levels via its transcription targets. In the beginning of the NF-κB activation process, it appears that Traf recruits the signaling complex. In resting cells, NF-κB attaches to inhibitory IκB proteins in the cytoplasm. Upon upstream signaling, such as EDA signaling, IκB is phosphorylated by the inhibitor of κB kinase (IKK). IκB is degraded, and then releases NF-κB, which is transported to the nucleus and activates many downstream molecules (Israel, 2000; Courtney et al., 2005). The EDA-EDAR- NF-κB pathway is rather complex, not straightforward. Furthermore, these components likely have multiple overlapping and complicated functions.

The TRAF (TNF-receptor–associated factor) signaling molecule is involved in TNFR activation of the NF-κB pathway. Generally, there are two distinct intracellular signaling mechanisms regulated by the TNFR member: direct and indirect binding to TRAF. Unlike many members of the TNF receptor family, EDAR fails to directly bind to any TRAFs (Headon et al., 2001; Yan et al., 2002). There are six members in the TRAF family (TRAF1 to -6). It is currently unclear that which members of the TRAF family involve in The EDA-EDAR- NF-κB pathway in ectodermal organogenesis. In order to clarify the roles of components in this pathway, the disruption of the EDAR-NF-κB signaling pathway was performed and the expression patterns of candidate gene and the phenotype of the affecting organs were examined. The first target gene in the EDA-NF-κB signaling pathway is *TRAF6*. TRAF6 mediates TAB2 (TGFβ-activated kinase 1-binding protein 2), which bridges TRAF6 to TAK1 (TGFβ-activated kinase 1) (Morlon et al., 2005). Upon TRAF6 activation, the IKK complex is stimulated to regulate the degradation of IκB. The expression of Traf6 in developing hair follicles also has been described (Naito et al., 2002). Interestingly, among all six members, only Traf6 deficiency results in an

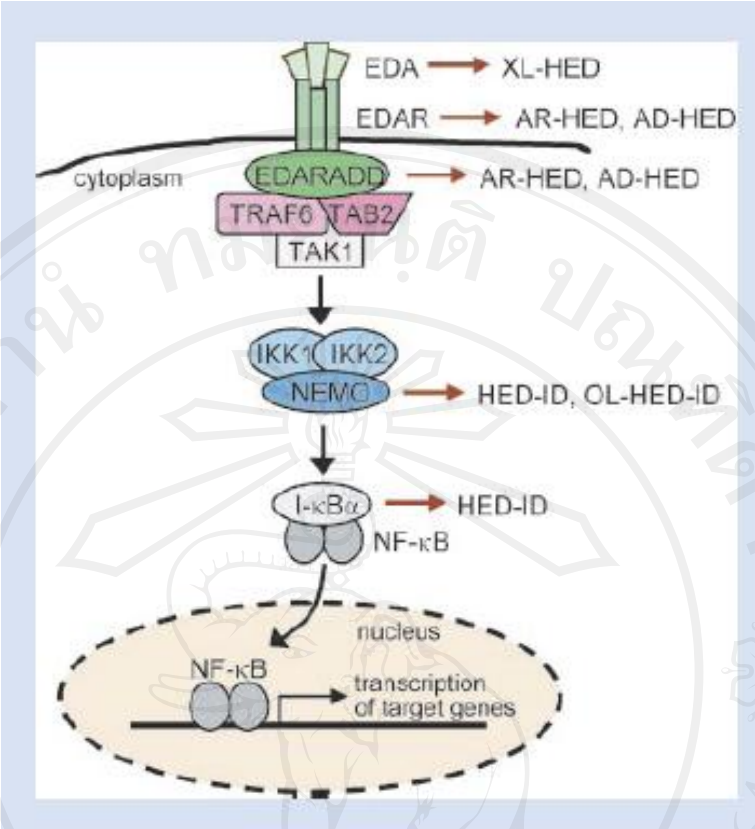

Figure 2.2. EDA-EDAR-NF- κB pathway. Upon upstream signaling of EDA activation, the soluble ligand binds with its receptor, Edar, resulting in NF-κB activation via the downstream molecules. Mutation within the components of the EDA pathway causes various types of hypohidrotic ectodermal dysplasia (Modified from Mikkola, 2009).

ectodermal phenotype resembling that of Tabby mice, which manifests several defects of epidermal appendages. Although Traf6-deficient mice have severally shared ectodermal defects with Tabby, Traf6 mutant mice often manifest more severe ectodermal defects than do Tabby mice. The Traf6 mutation affects bone and the immune system. This suggests that TRAF6 might be responsible for transducing the EDAR-NF-κB signaling, and could also involve another pathway besides the EDA pathway. However, no mutation of TRAF6 has been discovered in humans.

Furthermore it is currently unclear that TRAF6 mediation is a function of transducing the EDA signaling pathway in ectodermal organogenesis.

Although Traf4 is found throughout tooth development in both epithelium and mesenchyme, Traf4 knockout mice do not appear to have significantly developmental tooth defects. TRAF4 is not able to interact with EDARADD, which bridges EDAR to TRAF. Of the other TRAF molecules, TRAF2 displays a strong ability to interact with EDARADD, whereas TRAF5 is weak (Yan et al., 2002) and also not expressed during tooth formation (Ohazama et al., 2003).

TAB2 is an adaptor protein that bridges TRAF6 to TAK1. Activation of the ectodysplasin pathway involves holding of the TAB2 to EDARADD (Morlon et al., 2005). Upon upstream signaling, TAK1 is activated, followed by IKK activation. The IKK protein complex has an effect on IκB phosphorylation (Israel, 2000; Courtney, 2005). and is responsible for NF-κB signaling. The IKK complex is composed of two catalytic subunits (IKK_{α} and IKK_{β}) and an obligate regulatory subunit (NEMO or IKK_{γ}). Although IKK_{α} and IKK_{β} are highly homologous, they have their own functions and are unchangeable.

IKK knockout mice have been reported to die with quite different phenotypes. First of all, IKK_{α}-deficient mice die soon after birth with obvious limb and skin defects. The limbs of IKK_{α}-deficient mice are stagnant inside their skin because of a thickened and undifferentiated epidermis (Takeda et al., 1999). In contrast, IKK_{β}-deficient mice die with severe liver apoptosis, during the early embryonic stage, around embryonic day 13 (E 13) (Li et al., 1999). The other member of the IKK complex, NEMO, is required for NF-κB signaling, as are the two previously described members (Yamamoto et al., 2001). The mutations in NEMO also cause HED

phenotype and other symptoms not related to EDA mutation. Mutations in both humans and mice that completely abolish NEMO cause X-linked dominant incontinentia pigmenti (IP; OMIM 308300). Males with IP usually die before birth, whereas most affected females survive with wide-range phenotypic severity and typically manifest multiple skin lesions, blindness, neurological disturbances (Makris et al., 2000). The high variability occurred in female patients with IP has been attributed to the skewed pattern of X-inactivation. Furthermore, in humans, hypomorphic mutations of NEMO, which partially impair, and do not completely lose its function, can cause two related immune-deficiency syndromes; hypohidrotic ectodermal dysplasia with immunodeficiency (HED-ID; OMIM 300291) and osteopetrosis and/or lymphoedema with HED-ID (OL-HED-ID; OMIM 300301) (Doffinger et al., 2001)

In response to IKK upstream signaling, IκB turns to phosphorylated form. NF-κB, a transcriptional regulator, is released and then moves into the nucleus for transcription. There are a number of different IκBs, in which the exact molecules involved in ectodermal organ development are still to be proved.

2.1.4 Regulation of the expression and associations with other signaling pathways

The epithelial-mesenchymal interaction is known to implicate in development of all ectodermally-derived organs. The underlying mesenchyme seems to first initiate the signal to the overlying ectoderm for formation of epithelial thickenings, known as follicle placodes (Hardy, 1992). Further signaling from the placodes, which acted as transcient signaling centers, sends back and patterns to the mesenchyme (Schmidt-Ullrich and Paus, 2005). The communications between these two tissues induce

initiation, morphogenesis, and differentiaion of ectodermal organs at last. During hair follicle development, the Wnt/β-catenin and Eda/Edar pathways seem to be the earliest mechanisms (Schmidt-Ullrich and Paus, 2005). Wnt/β-catenin activation is detected in dermis at E13.5 before the presence of NF-κB signaling, which is observed in the epithelial placodes at E14.5 (Schmidt-Ullrich et al., 2006; Zhang et al., 2009). Several pieces of co-supporting evidence indicate that Eda is regulated by Wnt signaling (Figure 2.3), its abrogation completely blocks hair development (Andl et al., 2002) Wnt signaling is known to be a key regulator of the development of several ectodermal organs, including hair, feathers, teeth and mammary glands, in several stages of development.

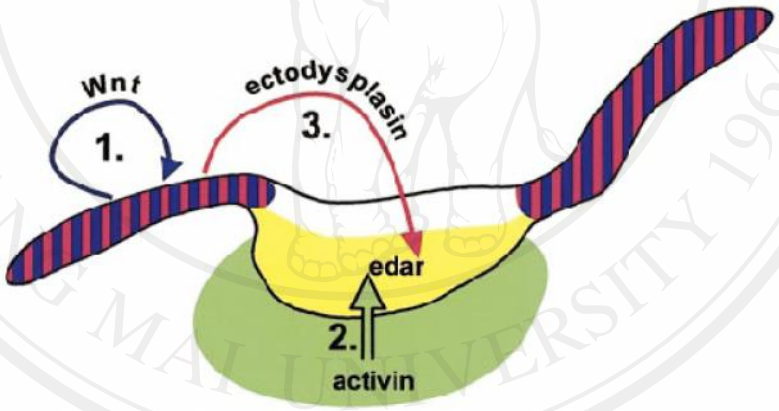

Figure 2.3. Schematic presentation of the regulation of expression of ectodysplasin and of Edar, associated with communication between ectodermal and mesenchymal tissues, and other signaling pathways, during early tooth development. The expression of Eda in the oral ectoderm is regulated via LEF-mediated Wnt signals, whereas the expression of Edar in the epithelial placode is regulated by Activin β in the underlying mesenchymal tissue previously induced by the epithelium signal. (Modified from Laurikkala et al., 2002)

To support the activity of Wnt/β-catenin signaling in ectodermal organ development, Dickkopf (Dkk) 1, known as Wnt inhibitor, which be used to find out a

role of Wnt/β-catenin signaling in hair follicle development, inhibits hair formation and expression of other required signaling molecules in ectodermal placodes of embryonic mice (Andl et al., 2002). Analysis of tooth cultured explant first revealed that Wnt6 induces the expression of Eda in the epithelium (Laurikkala et al., 2001). Later results from skin cultured explant have shown similar result to previous analysis (Laurikkala et al., 2002). In experimental mice (TOPGAL), the expression corresponding to Wnt signal activity is in the basal epithelial cell layer of hair pre-germs and subsequently in cells of dermal condensates, suggesting Wnt signal involves in the first steps of hair morphogenesis (Mikkola and Millar, 2006). In Lef-1 knockout mice, the expression levels of Eda are decreased in ectoderm. Correspondingly, Eda possesses a Lef1 binding site in the promoter region and binds to Lef1 as revealed by electrophoretic mobility assay (Kere et al., 1996; Srivastava et al., 1997). These support that Wnt signaling mediated by β-catenin/Lef/Tcf transcription factor is upstream of Eda.

After the onset of these two signaling pathways, their patterns of expression are overlapping and interdependence to control the initiation and the maintenance of hair follicle placodes (Zhang et al., 2009). The epithelial β-catenin requires for patterning of dermal Wnt/β-catenin signaling. Wnt/β-catenin signaling is absolutely required for NF-κB activation. According to the first signaling pathway of Wnt/β-catenin, its activation can occurs in the absence of Eda-A1/Edar/NF-κB signaling. Tabby, downless, and mice with suppressed NF-κB activity ($c^{I\kappa B\alpha\Delta N}$) are detected Wnt expression between E13.5 and E14.5, which is an unexposed time of Eda-A1/Edar/NF-κB signaling. Previous reports have identified the *Eda* and *Edar gene* as direct target of Wnt/β-catenin and required for hair and tooth initiation and

morphogenesis (Laurikkala et al., 2001; Durmowicz et al., 2002). However, either Eda-A1, or Edar cannot be rescued placode formation in the absence of Wnt/β-catenin signaling (Zhang et al., 2009).

Edar expression is confined to the developing ectodermal tissue and is first found during placode formation, but expression in the early ectoderm prior to placode formation has not been described. The skin and teeth of mouse explant culture experiments indicate that the TGFβ signal activinβA, signaling from mesenchyme underlying hair and tooth placode, stimulates *Edar* expression in the forming placodes in both tissues. However, activin could not induce the expression of Edar in isolated ectoderm, requiring the presence of mesenchyme to upregulate the Edar. Loss of function of activin leads to failure of tooth and hair development. Edar did not regulate its own expression and the expression of Eda. The downless mice could express the strong Eda signal and their teeth were normal (Tucker et al., 2000). Contrastingly, in Tabby mice, Edar is expressed in the epithelium of tooth enamel knots and in the hair follicles, not restricted to placodes as seen in wide type mice, this suggests that Eda signaling regulates the patterning of Edar expression (Laurikkala et al., 2001; Laurikkala et al., 2002).

2.1.5 Molecular targets of *EDA*

Despite the diversity in shape and function of ectodermal appendages, they share common morphology and molecular mechanism during the early steps of morphogenesis. Initially, they all develop their patterns as a result of the regulation of the balance of stimulatory and inhibitory signals between surface ectoderm and underlying mesenchyme (Hardy, 1992). The first visible sign of all ectodermal organ formation is a local thickening of the epithelium, the placode, which results from an

upstream signal from condensed mesenchyme. The epithelial-mesenchymal interaction or the communication between and within the two tissues is mediated by several families of signaling molecules, including Wnts, fibroblast growth factors (FGFs), transforming growth factors-β (TGF-βs), bone morphogenetic proteins (BMPs), and sonic hedgehog (SHH), which are displayed during early morphogenesis, and highly conserved in vertebrates (Pispa and Thesleff, 2003; Mikkola and Millar, 2006). Several different methods, such as the explant culture of ectodermal tissue, have been used to identify which molecules are downstream of EDA.

BMPs generally function as inhibitors of placode formation and control the patterning of hair follicles. Several different approaches have revealed that Eda has a role in suppressing Bmp activity and in regulating the expression of two BMP inhibitors: Ccn2/ctgf (connective tissue growth factor) and follistatin (Mou et al., 2006; Schmidt-Ullrich et al., 2006; Pummila et al., 2007). These gene were expressed in developing skin of *Eda* mutant and wild-type mice (Cui et al., 2006). In *Eda* deficient skin, suppression of BMP activity is impaired. In addition recombinant *Eda* was lead to antagonize the activity of BMP4 in developing tooth germ.

As previous described, Wnt signaling is an absolute requirement for initiation of several ectodermal organs. But during later stages of primary hair follicle development, Eda-A1/Edar/NF-κB signaling is conversely required for maintaining the conserved Wnt/β-catenin activity. The Wnt inhibitor Dkk4, identified as a target of Eda-A1 and Wnt itself, strongly localized with Edar in the placodes of ectodermal organs, and the recent study has shown that Dkk4 is necessary to regulate the placode formation by negative feedback mechanism (Fliniaux et al., 2008). During placode

formation, Eda also strongly induces the expression of Dkk4, a soluble Wnt inhibitor and a target of Wnts itself. The Lrp4 gene has been shown to be involved in the Wnt pathway. Upon Edar activation, the Lrp4 genes were found to colocalize with Edar as does Dkk4, but Dkk4 is stronger expression than Lrp4. However, low Dkk4 and Lrp4 expression is retained in the absence of NF-κB signaling in Eda null hair placodes. Dkk4 has recently been suggested as a key Wnt antagonist, essential for the normal pattern of hair follicles, as Lrp4 has been shown to be essential.

In situ hybridization study has shown that in mice with suppressed NF-κB activity ($c^{I\kappa B\alpha\Delta N}$), *wnt10b* expression was abnormally weak at E14. This suggests that pattern of Wnt10b expression is Eda/Edar/ NF-κB-dependent (Zhang et al., 2009). Similarly, expression of Wnt10a, close relative to Wnt10b, disappears in skin of these mutant mice. In humans, the absence of WNT10A function is associated with defective hair follicle development (Adaimy et al., 2007) with severe hypodontia too (Borhing et al., 2009).

Sonic hedgehog is another target gene of *EDA* (Pummila et al., 2007). Shh play a role in correct morphogenesis of hair development (Mikkola and Millar et al., 2006). The mammalian dental experiment has shown that Shh appears to absolutely downregulate in Tabby teeth (Kangas et al., 2004)

Lymphotoxin-β (LTβ) is a TNF-like ligand with previously identified roles in lymphoid organogenesis. LTβ is enriched in developing hair follicles of wild-type, and Eda also induces the expression of LTβ and when loss of Eda signaling, LTβ is absence. Furthermore, in LTβ-deficient mice, all types of mouse hair pelage are still formed, but their structure is abnormal. Thus, as an EDA target, LTβ regulates hair

differentiation and may partially explain hair fiber abnormalities when EDA is defective (Cui et al., 2006).

2.2 Ectodermal organogenesis

2.2.1 Expression pattern

Eda, Edar, Edaradd are expressed in the epithelial cells of developing ectodermal organ including teeth, hair follicles, and eccrine sweat glands during embryonic development. The expression of ectodysplasin and Edar has been analyzed during murine hair and tooth development, the results of the expression depending on the type of tissue and the analysed technique. Eda and Edar are restricted to the epithelium. Despite both isoforms of Eda (EDA-A1 and EDA-A2) have biological function, only Eda-A1 is expressed in the early epidermis, whereas Eda-A2 is not (Yan et al., 2000). In general, the Eda and Edar patterns are similar and co-localized in early developing ectoderm. When placodes are formed, the Eda and Edar patterns are different and separated. Edar is expressed in the placode and downregulated in the interfollicular epidermis, whereas Eda displays complementary expression in the interplacodal ectoderm and becomes downregulated in the placodes (Figure 2.4) (Headon and Overbeek et al., 1999; Tucker et al., 2000; Laurikkala et al., 2001; Laurikkala et al., 2002). Edaradd has been detected in the same cells as Edar (Headon et al., 2001).

During later stages of hair development, Both Eda-A1 and Edar are confined to the bulb region, where the precursor cells for hair follicles reside (Mikkola et al., 1999; Yan et al., 2000; Laurikkala et al., 2001). In addition, Eda-A1 expression is remained and also detected in the interfollicular epidermis responsible for the initiation of zigzag and auchene hair follicles (Yan et al., 2000). During advanced

development of molar, Edar is specifically expressed at the tip of the bud, which later forms the enamel knot and responsible for crown morphology (Jernvall and Thesleff, 2000). On the other hand, expression of Eda is excluded from the sites of Edar expression and becomes confined to the outer enamel epithelium (Pispa et al., 1999; Laurikkala et al., 2001).

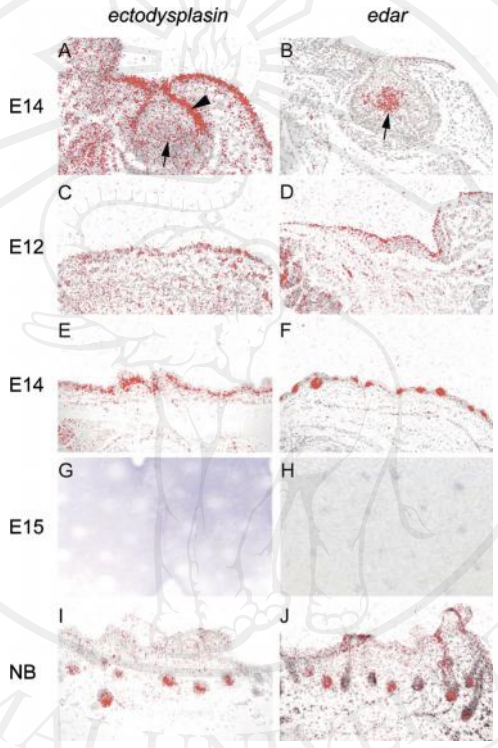

Figure 2.4. Non-overlapping expression pattern of *Eda* and *Edar* during ectodermal organogenesis. The expression of *Eda* and *Edar* in murine tooth and hair is restricted to the epithelium. In the cap stage, tooth germ expression of *Eda* and *Edar* is different. Eda (A) is confined to the outer enamel epithelium (arrowhead) (E14) whereas *Edar* (B) is strongly expressed in the signaling center at the enamel knot (arrow). During early hair follicle development *Eda* and *Edar* transcripts are colocalized in the developing ectoderm (C and D). Upon hair germ initiation, their expression pattern is altered. *Edar* is strongly displayed in the placodes (F and H), but contrast to *Eda* which remains to be expressed in the interfollicular epithelium (E and G). At later stages (I and J), *Eda* and *Edar* expression return to co-express in the bulb of the hair follicles of the newborn (NB) and some expression is also detected in the epidermis (Modified from Laurikkala et al., 2001).

In addition, northern blot analysis and in situ hybridization from human fetal tissue have revealed that Eda is also expressed in various organs, not only in ectoderm but also in mesoderm and endoderm such as heart, brain, lung, liver, small intestine, kidney, and anterior pituitary (Kere et al., 1996; Bayes et al., 1998). Edar is also expressed in lung and kidney and during early limb development in the apical ectodermal ridge (Tucker et al., 2000). A preponderance of evidence suggests that ectodysplasin signaling is necessary for development of ectodermally-derived organ, especially in their initiation, morphogenesis and differentiation stage. The function of Eda-A1 appears to be downstream of the primary inductive signal required for placode initiation during skin patterning (Mustonen et al., 2004).

2.2.2 Stages of ectodermal organ development

Development of all ectodermal organs, such as hairs, feathers, scales, nails, teeth, sweat glands, and mammary glands whose origins are both epidermal or oral ectoderm and mesodermal mesenchymal (hair and mammary gland) or neural crest derived mesenchymal (tooth) tissues, is regulated by a reciprocal sequence of inductive epithelial-mesenchymal interactions (Thesleff and Mikkola, 2002). Despite the great diversity in shape and function, ectodermal organs generally share several common features and molecules in their early steps of development, especially in early morphogenesis (Mikkola and Millar, 2006). The epithelial-mesenchymal interactions constitute by many reiterative signals via signal molecules of the two tissue layers and regulate multiple stages of developmental functions yielding initiation, proliferation, morphogenesis, and differentiation (Mill et al., 2003; Schmidt-Ullrich et al., 2006). Generally, the mesenchymal tissue seems to provide the first instructive signal, which is followed by the feedback of signaling from the

ectodermal tissue. The formation of the epithelial placode is the first sign of all developing ectodermal organs. That placode is a thickening of the epithelial layer that results from the inductive mesenchymal signaling giving the epithelium cells aggregate each other, and act as an early signaling center providing the signal back to the underlying mesenchymal cells for further interactions. The overlying placode projects into or out of the mesenchyme, and subsequent proliferation, cell migration, and contributes to morphogenesis (Figure 2.5). The epithelium becomes bud-shaped which seems to be highly similar in many ectodermal appendages. In the transition of placode to bud, the interaction between both layers has continued. After the bud stage, the anatomy of each organ becomes prominently different and depends on the growth pattern of each organ.

In hair follicle development, epidermal keratinocytes, which response from the first epithelial-mesenchymal interaction, are plays a role to cluster together and originate placode. Each placode returns the signal to the underlying dermis, resulting in the formation of a mesenchymal cell aggregation known as the dermal condensate. The epithelial cells extend downward to wrap around the condensed mesenchymal cells known as the dermal papilla. During the differentiated stage of hair development, the epithelial matrix cells, contracting the dermal papilla, give rise to the inner root sheath (IRS). The layers of IRS surround the three contact layers of hair shaft, medulla, cortex, and cuticle. Finally, the whole follicle is enveloped by an outer root sheath (Schmidt-Ullrich et al., 2005). There are four mouse pelage hair types, which develop from different successive waves. The primary hair follicles initiate the long guard hairs (E14), the second wave initiates the shorter awls (E17), and the two last types, the auchenes and zigzags originate from placodes formed

before birth. The hair follicles continuously mature. During new hair cycling, the hair is initiated from the epithelial stem cells in the bulb region (bulge), not from the ectodermal matrix cells which normally originating the developing hair follicle, by stimulating of cells resided in dermal papilla.

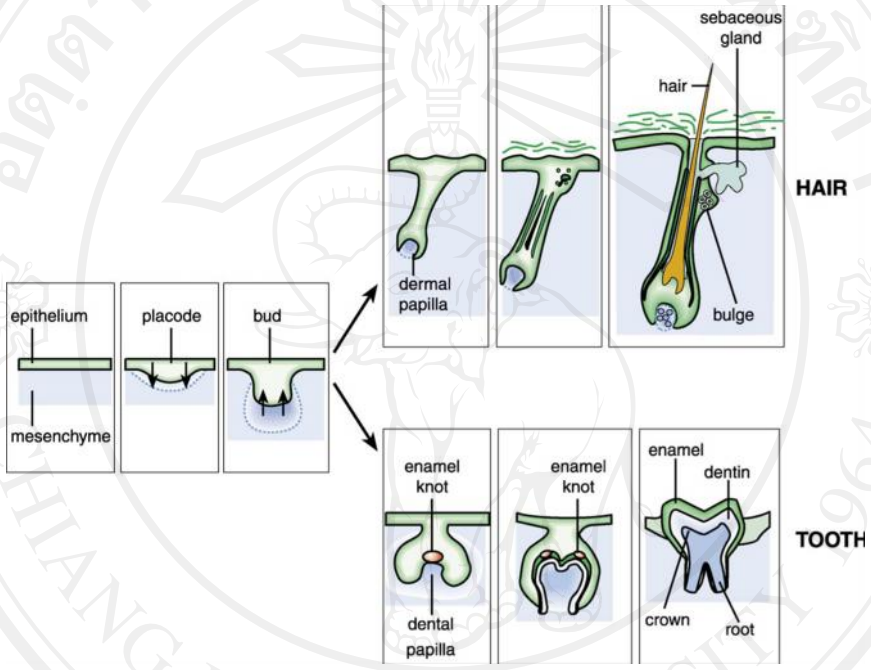

Figure 2.5. Schematic presentation of the development of hair and tooth. Both hairs and teeth originate from surface epithelium and underlying mesenchyme. Their development is regulated via the numerous signaling molecules mediated from these two tissues. The early stages share a common morphology as the ectodermal placodes extend to the buds and then induce the condensation of underlying mesenchyme, which further form as the dermal and dental papillae. In the later stages the morphology and differentiation of hair and tooth are different. The developing hair shaft is formed by ectodermal cells but new hair cycling originates from stem cells in the bulge. In the tooth germ, the enamel knot signaling center appears at the tip of the dental epithelium and later forms the secondary enamel knot, determining the cusp morphology of the tooth. (Modified from Mikkola and Thesleff, 2003)

Mice have one incisor and three molars on each side, a diastema or dental space acting as a separator between these types. During the tooth development, there are three morphogenetic stages: bud, cap, bell stage as those seen in hair follicle development. Formation of tooth occurs at around E11 in each side of mouse jaw (Jernvall and Thesleff, 2000). Between E12 and E13, the dental placode grows downward and invaginates into the underlying condensed mesenchyme and subsequently form the bud of tooth germ. At the end of tooth bud stage, epithelial cells at the tip of such bud cease proliferation and transform to the enamel knot which is a crucial signaling center containing many signaling molecules regulating specific tooth morphology. At around E14-E15, the epithelial cells laterally beside to the enamel knot rapidly grow extensively into the dental papilla that turn the development to the cap stage. The primary enamel knot disappears by apoptosis around E16, and was replaced by the secondary enamel knots which function in patterning of cusps in molars. At the late bell stage, dental papilla cells differentiate into dentine-producing odontoblasts, while the epithelial cells (inner enamel epithelium) give rise to enamel-producing ameloblasts. As in the hair follicle, crown and root continue to develop gradually and the tooth sequentially erupts after birth.

Among different ectodermal appendages of mouse, mammary gland development starts first at around E10.5 (Veltmaat et al., 2003; Chu et al., 2004; Veltmaat et al., 2004; Hens and Wysolmerski, 2005). The epithelium which gives rise to future mammary lines appears and thickens. There are five pairs of mammary placode in mouse (Veltmaat et al., 2003). Each mammary placode is emerged upon the mammary line between E10.5 and E11.5 and then invaginates into condensed mesenchyme and transforms into bulb-shaped bud. During the later stage of

development, the formation of the nipple and the branching morphogenesis of premature mammary glands occur and mature after hormonal stimuli during puberty or pregnancy.

2.2.3 Role of EDA in ectodermal organogenesis

The interactions between epithelium and mesenchyme are mediated by many signaling molecules that commonly shared between the different ectodermal appendages (Millar, 2002; Thesleff and Mikkola, 2002). The signaling is expressed reiteratively during the development of each ectodermal organ such as Wnt family, FGFs, BMPs, TGFβ, and SHH. In addition, the ectodysplasin and its receptor are also expressed in the developing organ appendages and are accompanied by other signaling molecules, as described above.

The ectodysplasin pathway has an important function in embryonic development and is highly conserved from fish to human, especially in the development of ectodermally-derived organs in vertebrate species (Pantalacci et al., 2008). These similar phenotype in ectodermal organs were caused by mutation that interrupts NF-κB activation. Mutations interrupting NF-κB activation display similar phenotypes in ectodermal organs (Mikkola and Thesleff, 2003; Schmdt-Ullrich et al., 2001).

All patients with HED and mice with mutations in the components of the Eda pathway display an identical ectodermal dysplasia phenotype, lack the first hair placodes and impaired developments of teeth, sweat glands and mammary glands (Headon and Overbeek, 1999; Laurikkala et al., 2002), whereas in transgenic mice, overexpression of *Eda* induces ectopic teeth and mammary glands, and shortens the growth cycle of hair and increases the activity of sweat glands (Mustonen et al.,

2003). Although there are species-specific differences and compensatory pathways between mice and humans, their ectodermal dysplasia phenotypes are identical. The results of experiments in mice, either deficient in or overexpressing Eda, have revealed the function of Eda in embryonic development (Courtney et al., 2005). A better understanding is needed of the role of EDA in ectodermal development in humans.

(1) Consequences of the loss of Eda signaling in mice

The pathogenesis of defective ectodermal organ development has been studied in all four types of spontaneous mouse mutant model, mainly in Tabby mice, and mostly in hairs and teeth. However, all mouse models with loss of Eda signaling seem to be identical phenotypes. The typical features in mouse mutants include missing or malformed teeth, defective hair and reduced sweating ability similar to what is seen in humans. In the dental field, about 20% of Tabby mice lack incisors and/or third molars (Pispa et al., 1999) (Figure 2.6). The patterning of Tabby molar crowns is abnormal. This results from impaired signaling in the enamel knot, which regulates the cusp patterning (Pispa et al., 1999). The cusp of Tabby seems to be flattened and the size of tooth arch smaller compared with the wild type. Tucker and Sharpe suggest that Eda signaling regulates the initiation of tooth formation and the function of the enamel knot, which is known to be an organizing center regulating the shape of teeth (Tucker and Sharpe, 2004). In Eda null mice, the enamel knot is altered to small in size and decreased its growth factors, suggesting that Eda regulates tooth development via enamel knot.

The lack of the first hair wave, which, normally, develops into the guard hairs, is an obvious phenotype and always occurs in *Tabby* mice (Laurikkala et al., 2002)

(Figure 2.6). The lack of the first hair wave indicates that ectodysplasin signaling is responsible for the formation of the primary hair follicle, which develops in successive waves, starting at E14. The expression of Edar is abnormal. It is expressed in epithelium where normally be absent of Edar expression. In addition, these mice also miss the last hair wave, which, normally, develops into the zigzag and auchene hairs. Thus tabby mice have only the abnormal awl hair type.

The number of sweat glands on the footpad of mice, using iodine starch testing, is decreased compared with those in wild type mice (Mustonen et al., 2003). However, mice without ectodysplasin signaling do not inhibit organogenesis completely. It is likely that some other pathways directly and crucially compensate in organogenesis.

(2) Stimulation of ectodermal organ development by Eda

Under the action of the keratin 14 (K14) promoter in transgenic mice, overexpression of *Eda-A1* resulted in alteration in several ectodermal organs (Mustonen et al., 2003; Mustonen et al., 2004) (Figures 2.6 and 2.7). Transgenic mice have supernumerary teeth, which occur in the diastema separating the incisor area from the molar area. These supernumerary teeth are smaller than normal mouse molars. The abnormality in cusp pattern of these teeth is not significantly detectable. Besides, the mandibular incisors have an enamel hypoplasia phenotype, leading to severe attrition and supraocclusion of the opposing teeth. The hair of transgenic mice is affected, not only in hair type, but also in length and composition. Eda has been known to control hair follicle cycling, therefore the transgenic hair growth develops continuously from E14 until birth and the growth cycle of the hair is prolonged, resulting in greater hair length than in wild type mice (Figure 2.7). Histology shows

that each hair follicle of transgenic mice is closely localized with others and hairs are fused together. Auchene and zigzag hair types are not detected in these mice. Supernumerary mammary glands develop and appear in the mammary line of transgenic mice. Another ectodermal organ, sweat glands are increased in number andfunction in transgenic mice and sebaceous glands are also enlarged, but there are no extra glands. Histology of hair and tooth placodes of K14-*Eda-A1* transgenic embryos has been shown that these placodes are irregular, enlarge and closely localized with others. These variations may result from either the stimulated growth or the fusion of placode. However, Eda-A1 recombinant protein does not rescue the initiation of the first wave of hair follicles in the *Eda* mutant mice (Mustonen et al., 2004), but sufficiently rescues the pattern of hair growth (Srivastava et al., 2001). In conclusion, Eda-Edar signaling has several roles in ectodermal organ development, controlling its initiation, as well as morphogenesis and differentiation in embryonic development.

2.3 *EDA*-associated human disorder

2.3.1 X-linked hypohidrotic ectodermal dysplasia

Ectodermal dysplasias (EDs) represent a large and complex group of diseases comprising approximately 200 different clinical conditions (Pinheiro and Freire-Maia, 1994; Priolo and Lagana, 2001; Lamartine, 2003; Itin and Fistarol, 2004). They are caused by impaired development of epidermal appendages and characterized by a primary defect in the following tissues: hair (hypotrichosis, partial or total alopecia), teeth (abnormal or absent), sweat glands (hypoplastic or aplastic) and nails (dystrophic, hypertrophic, abnormally keratinized). Several classifications of ED have been proposed, base on different criteria, such as clinical (Pinheiro and Freire-

Maia, 1994) and molecular genetic findings (Priolo and Lagana, 2001). The causative gene has been identified for about 30 types of ED.

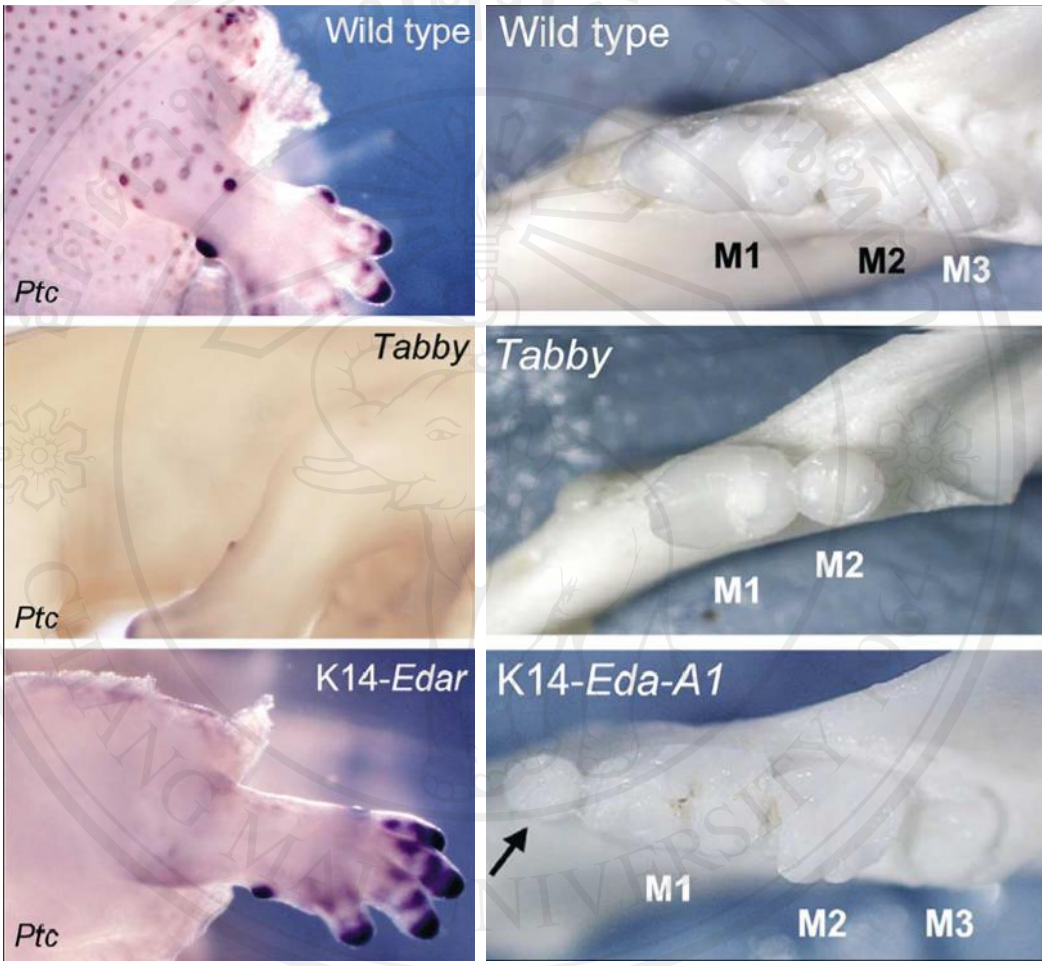

Figure 2.6 Comparison of primary hair follicle of wild type, *Tabby*, and K14-*Edar* and molars of wild type, *Tabby*, and K14-*Eda-A1* mice. The development of primary hair follicles is inhibited in Tabby as well in K14-Edar embryonic mice, but not in wild type mice. Focus on dental features, wild type mice have three molars. *Tabby* mice often lack the third molar, and the size and number of cusps of first and second molars is altered. In K14-*Eda-A1* mice, a supernumerary tooth (arrow) appears in the diastema. This extra tooth is small and irregular in shape (Modified from Mikkola and Thesleff, 2003).

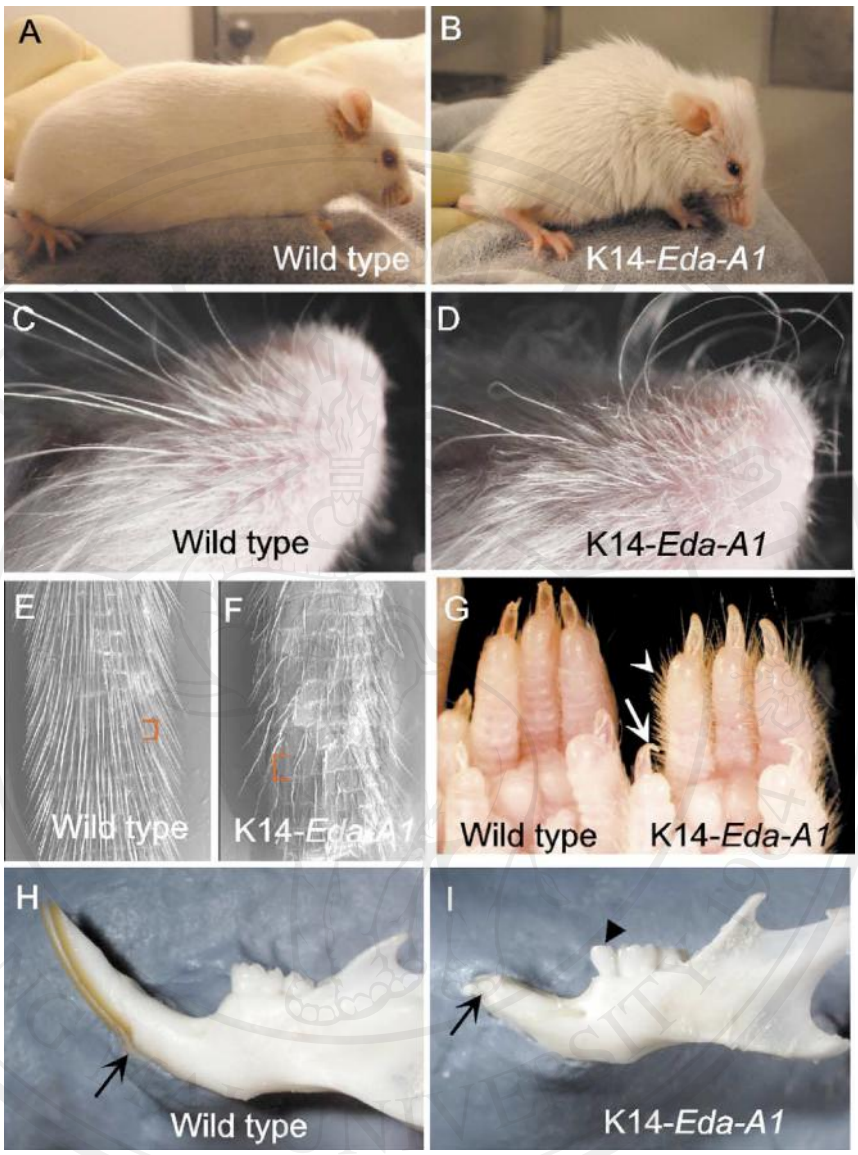

Figure 2.7 Phenotypic comparison of wild type, and K14-*Eda-A1* mice. Stimulation of *Eda-A1* affects the development of several ectodermal organs. (A, B) K14-*Eda-A1* mice show a distinct appearance from the wild type; its hair is untidy. (C–F) Similar to body hair, the whiskers (D) and tail hairs (F) of two K14-*Eda-A1* transgenic lines are curly as compared to those of wild type mice (C,E). SEM shows that the scale-like folds of tail skin of all transgenic mice are larger (brackets in E and F). (G) Nails (arrow) and hairs (arrowhead) in the feet of transgenic animals are longer. (H, I) K14-*Eda-A1* lower incisors have severe attrition (arrows in H and I indicate the bone margin). In addition, a supernumerary molar (arrowhead) is seen in the transgenic lower jaw (Modified from Mustonen et al., 2003).

Hypohidrotic ectodermal dysplasias (HED), a common group of ectodermal dysplasias is a rare congenital disease with an estimated incidence of 1 in 100,000 births (Stevenson and Kerr, 1967; Zonana, 1993). The hypohidrotic ectodermal dysplasias (HED) form, first characteristically described by Thurnam in 1848 (Thurnam, 1848) and later by Darwin in 1875 (Darwin, 1875), involves all of the Mendelian modes of inheritance (Pinheiro and Freire-Maia, 1994; Lamartine, 2003). The most prevalent form of HED is X-linked hypohidrotic ectodermal dysplasia (XLHED; OMIM 305100), whereas the autosomal forms are found with a lower frequency (Headon et al., 2001; Shimomura et al., 2004; Chassaing et al., 2006; van der Hout et al., 2008). Molecular etiologies of HED consist of mutations of the genes involved in the EDA-NF-κB pathway. The X-linked form of HED corresponds to mutations in the TNF ligand ectodysplasin (Kere et al., 1996; Monreal et al., 1998; Schneider et al., 2001; Vincent et al., 2001). Both autosomal-dominant and -recessive forms of HED involve *EDAR* and *EDARADD* mutations (Chassaing et al., 2006; van der Hout et al., 2008). Syndromic HED with immunodeficiency and/or osteopetrosis is associated with mutations in NF-κB essential modulator IKKγ (*NEMO*).

This is a unique spontaneous example of mutations in ligand, receptor, or adaptor giving rise to the identical phenotype characterized by defects in the development of ectodermal appendages presumably due to NF-κB response. Intra- and Inter-familial variability exists in degree of clinical feature of HED. The typical signs of HED include missing or sparse hair (atrichosis or hypotrichosis), missing teeth (anodontia or hypodontia), in either the primary or permanent dentition, and absent or reduced numbers of sweat glands and consequently, loss or reduction of their function (anhidrosis or hypohidrosis), occasional dystrophic nails and hypoplasia

of mammary glands. It is detectable that the reduced sweating results in severe hyperthermia which can cause death in affected children, if it is severe and undiagnosed in early life. Other abnormalities commonly found in HED, are dryness of the skin, eyes, and other mucous membranes. The sparseness occurs in body hair and eyebrows, but facial and pubic hair is usually normal. Facial appearance is unusual, with frontal bossing, depressed nasal bridge and everted lips. There are often linear wrinkles and pigmentation around the eyes (Figure 2.8).

Among the ectodermal appendages associated with *EDA* mutation, dental tissue is the most highly affected. Hemizygous males have multiple missing permanent teeth. Excepting the third molars, the mean number of missing teeth in affected males is 22 whereas that in female carriers is four, but both incidences show a higher frequency than that of normative data (Lexner et al., 2007). The most stable

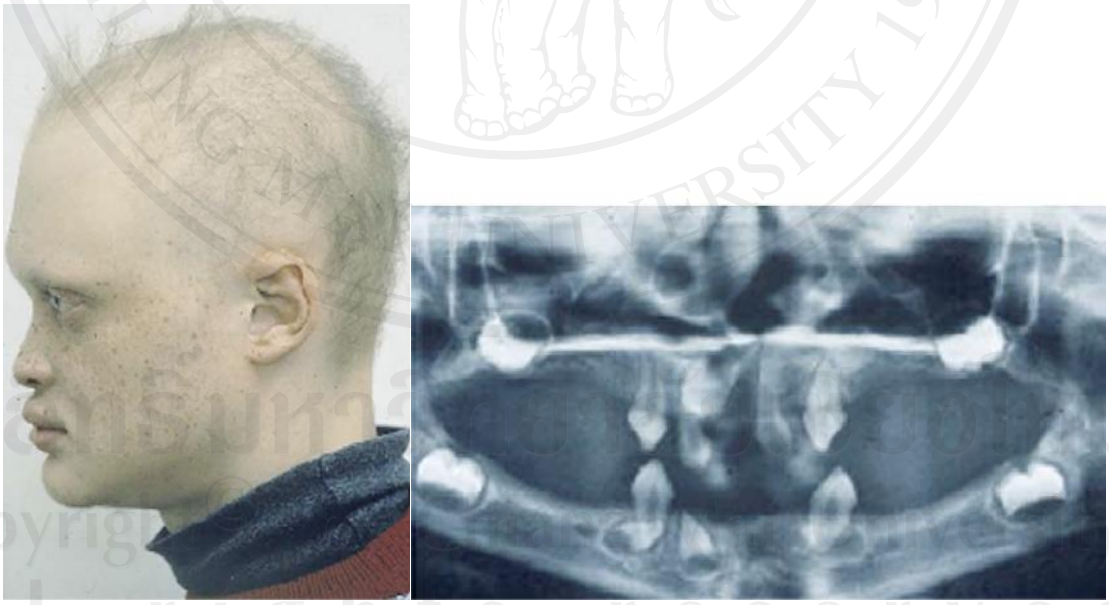

Figure 2.8 Phenotypic features of hypohidrotic ectodermal dysplasia. It includes sparse hair, absent eyebrows, hyperpigmentation, saddle-nose, and prominent forehead. At right is a radiograph showing severe hypodontia. (Modified from Mikkola and Thesleff, 2003**)**

teeth in affected males are the maxillary central incisors, the maxillary and mandibular first molars, and the maxillary canines. The teeth most frequently missing in female carriers are the maxillary lateral incisors, as in normative data. In addition, both hemizygous males and heterozygous females have increased prevalence of tooth malformations and reduced tooth size, which can occur in either crown or root. The tooth malformations include conical or tapered crowns, partially fused molar roots, and pyramidal roots (Lexner et al., 2007; Lexner et al., 2008).

Whereas hemizygous males manifest severe expression and consistent phenotypes, heterozygous female carriers have varying degree of abnormalities, ranging from asymptomatic to clinical hypodontia, hypohidrosis, hypotrichosis, and unilateral hypoplasia of the breast and often demonstrate mild to moderate form. Clinical detection of carrier based on physical examination, is problematic because of the process of X inactivation (lyonization), leading to mosaic pattern of functionally active and inactive progenitor cells, and subsequently resulting in wide-range clinical involvement. One in 500 females with hypodontia in the permanent dentition and one in 50 with hypodontia in the deciduous dentition may be a carrier for HED (Sofaer, 1981).

The clinical phenotype of patients with the autosomal recessive form of HED is indistinguishable from that seen in males with the X-linked recessive form (Munoz et al., 1997). The major clinical difference between the autosomal and X-linked recessive forms is the occurrence of complete manifestations in affected females. Because affected females with ARHED (OMIM 224900) are usually more severe phenotype than female carriers of XLHED. Few families with the autosomal dominant form of HED (ADHED; OMIM 129490) have been identified. The

phenotype of affected individuals seems to be less severe than that of individuals with X-linked inheritance. All affected patients of XLHED have reduced sweating, a fine, and sparse scalp and body hair, and missing some deciduous and permanent teeth. The maxillary incisors are frequently conical (Crawford et al., 1991).

In Thailand, Sungthong (สังข์ทอง) was an actor who had the typical appearance of HED. This is the code name of the actor who was famous in the era of 1970-1980 (figure 2.9) He had a strange look and problems on sweating, chewing, and. pronunciation due to lack of teeth. A brother (สังข์เทพ) is affected with this disorder. Sungthong had an unaffected son. Sungthong died in a car accident at the age of 37. Thai people have known about this disorder because of him.

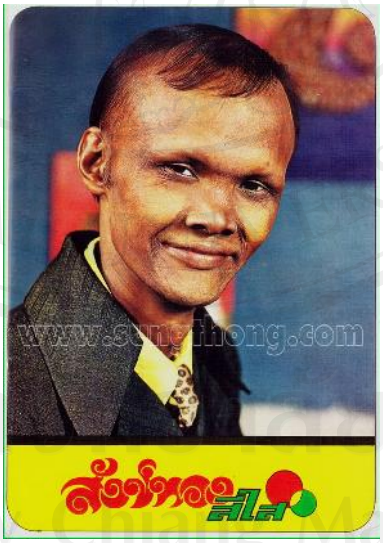

Figure 2.9 A photo of Sungthong, a Thai actor and singer. Due to his distinctive physical appearance, a patient, whose appearance is similar to him, is called as his name of Sungthong (http://www.sungthong.com).

2.3.2 Non-syndromic hypodontia

Missing teeth, formally named hypodontia, is a common craniofacial anomaly. Hypodontia is frequently seen in a large number of syndromes associated with other abnormalities such as ectodermal dysplasia and orofacial clefts. Hypodontia is also a major symptom in HED patients. However, hypodontia can occur as an isolated condition which named non-syndromic hypodontia. Several groups have intensively searched for other gene mutations causing non-syndromic tooth agenesis. To date, at least five genes, *PAX9* (Klein et al., 2005; Kapadia et al., 2006), *MSX1* (van den Boogaard et al., 2000; Mostowska et al., 2006), *AXIN2* (Callahan et al., 2009), *WNT10A* (Kantaputra and Sripathomsawat, 2010) and *EDA* have been identified to be associated with non-syndromic hypodontia. Among these causative genes, only the EDA gene is associated with the X-linked mode of inheritance when mutated. Interestingly, missense mutations in *EDA* have only been reported to be associated with isolated hypodontia without other abnormalities (Tao et al., 2006; Tarpey et al., 2007; Fan et al., 2008; Han et al., 2008; Li et al., 2008; Rasool et al., 2008).

As previously described, many evidences have indicated that *EDA* mutation is associated with the hypodontia phenotype in HED and isolated hypodontia. No correlation has been found between the location of the mutation and the severity of the phenotype. Only p.Asp316Gly mutation has been reported to be associated with both XLHED and non-syndromic hypodontia in the unrelated families (Li et al., 2008; Lexner et al., 2008; Zhang et al., 2011). This missense mutation affected the TNF homology domain which is responsible for the receptor binding. Interestingly, male affected with isolated hypodontia whose family was affected with XLHED has been found p.Ser374Arg mutation (Schneider et al., 2001). This mutation could

surprisingly cause not only XLHED, but also isolated hypodontia. However, the molecular pathogenesis of that mutation has not been clearly known.

To date at least 12 mutations of the EDA gene have been identified in non-syndromic hypodontia. The pattern of non-syndromic hypodontia caused by EDA mutations is not uniform. Often first permanent molars have not been affected and maxillary incisors are conical-shaped.

2.4 Previously reported *EDA* mutations

Molecular testing can help in diagnosing the type of XLHED disorders with which an individual is affected, in diagnosing carriers of XLHED, in appropriate medical management and in genetic counseling. Sporadic females with isolated hypodontia may be carriers of XLHED (Sofaer, 1981). Molecular testing is available for *EDA*. Exon 3, 5, and 9 which frequently recurred mutations should be screened first, followed by others. In addition to diagnosing and counseling, these mutations have proven to be informative in the analysis of the structure-function relationship of EDA. Over 100 families with presumed XLHED have been searched, for more than a decade, mutations in *EDA*, and numerous mutations have been successfully identified (Paakkonen et al., 2001; Schneider et al., 2001; Vincent et al., 2001; Hashiguchi et al., 2003).

As previously described, mutations in *EDA* can cause both HED syndrome and non-syndromic hypodontia. Mutation detection rates vary from 63% to 95% (Monreal et al., 1998; Schneider et al., 2001; Vincent et al., 2001), depending on the detection technique and on case selection. Direct sequencing results in the highest detection rate of mutations. All types of EDA mutations, such as missense, deletions, insertions, and nonsense mutations, have been noted (Figure 2.10). Large genomic

rearrangements with gross deletion also occurred. Missense mutations are the most frequent. Mutations in any part of *EDA* were found and could cause HED.

The functional structure of EDA was identified in three regions: the C-terminal TNF homology domain, the collagen-like domain, and a furin protease recognition sequence (Schneider et al., 2001). Mutations in the TNF domain, which is responsible for Edar binding ability, are scattered round this whole region. Mutations in this region affect the binding ability of EDA to EDAR. However, some mutational loci are severely effect on this ability, but some are weak. Patients with TNF domain mutation are differentially effect on clinical phenotype, extreme ranging from only dental defects to the three-symbolic feature of HED.

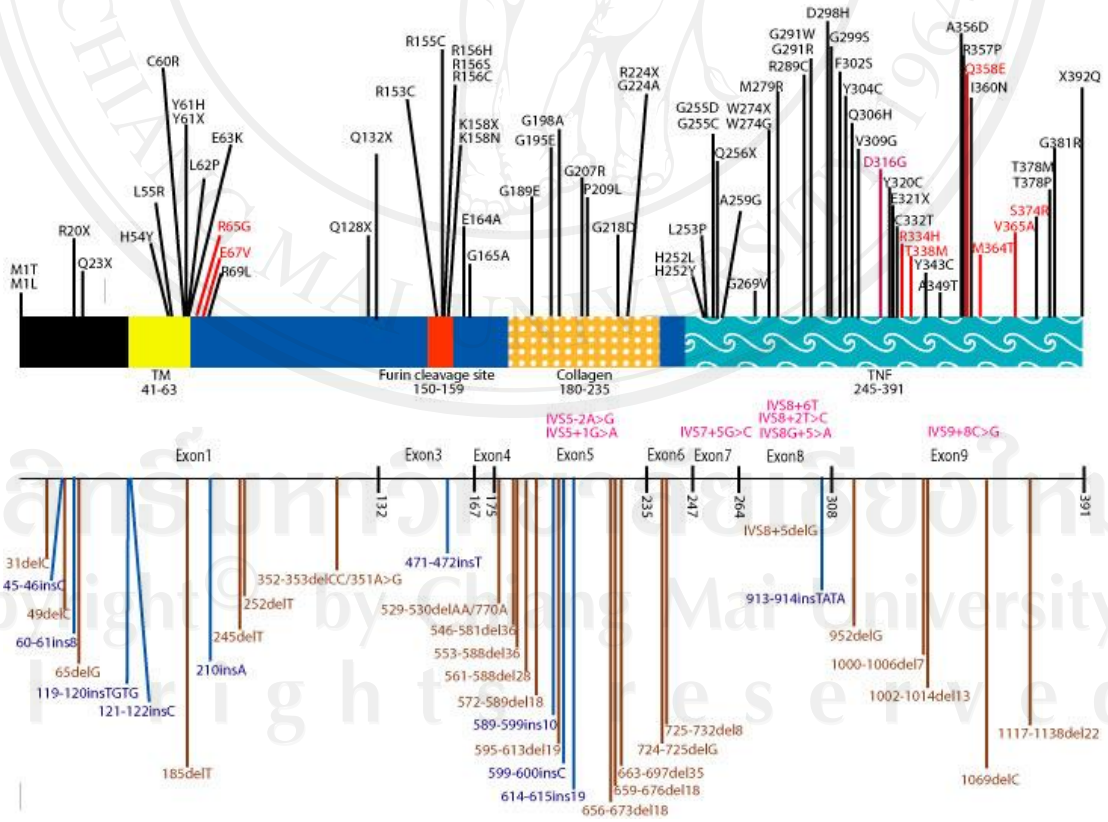

Figure 2.10 Pathologic variants of *EDA* from previous reports.

Different missense mutations, which altered the conserved glycine residues in the collagenous domain, have been reported. This region has a role in inducing multimerization of EDA trimers, not in receptor binding ability. Mutations in the collagenous domain, most containing exon 5, were frequently in-frame deletions and missense mutations (Schneider et al., 2001; Vincent et al., 2001).

Several groups have verified that EDA is shed from the cell surface and mutations in the furin recognition sequence significantly impair the cleavage processing (Chen et al., 2001; Schneider et al., 2001). Analysis of the reported variants in previous studies has shown a significantly non-random distribution of the mutations within the sequence of EDA. The most common of mutations identified in *EDA* occurs in the codons for the protease cleavage site within exon 3. These mutations are represented 20%-30% of mutations found (Schneider et al., 2001; Vincent et al., 2001). This largest cluster of mutations was responsible for only XL-HED, not non-syndromic hypodontia. That means this region is sensitive to mutate and play a significant role in ectodermal development. Mutational hot spot is constituted by a CpG-rich sequence which codes for two overlapping consensus cleavage sites (Arg153-Arg156 and Arg156-Arg159). Mutation at Arg156, affecting both cleavage sites, results in significant inhibiting of the proteolytic processing (Chen et al., 2001). Another recurrent missense mutation within the protease cleavage site, p.Arg153Cys, also impairs the normal processing, but its impact is less than Arg156 by forming an interfered disulfide bridge. In addition to coding regions, a number of mutations in splice-site and promotor regions have been identified (Kobielak et al., 1998; Schneider et al., 2001).

2.5 Genotype-Phenotype correlation

The phenotypic expression in XLHED results from impaired development of various ectodermal appendages and is characterized by defects in following tissues: hair (hypotrichosis), teeth (hypodontia) and sweat glands (hypohidrosis). Defects of nails (dystrophy, hypertrophy, abnormal keratin) may be seen in some patients. The severity of each affected organ varies and appears unpredictable (Schneider et al., 2001; Vincent et al., 2001). Many studies have shown that mutations in affected males cause only isolated hypodontia without affecting other organs (Tao et al., 2006; Tarpey et al., 2007; Fan et al., 2008; Han et al., 2008; Li et al., 2008; Rasool et al., 2008).

To date, all mutations associated with both XLHED and non-syndromic hypodontia are missense mutations and have occurred in the only TNF homology domain. Presumably, the wide range of phenotypic severity would result from the mutation within the TNF domain. Some patients have the mutation in the same locus, but the severity of their phenotype is different, and vice versa. Only two previous studies shown mutations associated with both XLHED and isolated hypodontia. First, p.Ser374Arg was found in a family with XLHED, which had two affected male and an affected grandfather with only hypodontia (Schneider et al., 2001). The other one was p.Asp316yGly, which associated with non-syndromic tooth agenesis and XLHED in the unrelated families (Lexner et al., 2008; Li et al., 2008). As the results from various studies that focused their attention to only XLHED patients, their findings indicated that no apparent genotype-phenotype correlation exists in those patients (Paakkonen et al., 2001; Schneider et al., 2001; Vincent et al., 2001; Hashiguchi et al., 2003; Lexner et al., 2008).

However, there probably exists a correlation between the phenotypes and genotypes comparing XLHED with non-syndromic hypodontia subjects (Zhang et al., 2011). Notably, hypodontia was more severe in XLHED than in isolated hypodontia patients. Comparing the number of missing teeth in XLHED with those in non-syndromic hypodontia, there were various statistically significant differences. First, the average number of hypodontia in XLHED was almost 22 compared with approximate 11 in non-syndromic hypodontia, this accounts for 76% and 38% of missing teeth in XLHED and non-syndromic hypodontia patients, respectively. Second, focusing on positions of missing teeth, XLHED had mainly missing at premolars or molars rather than anterior teeth. Although anterior teeth were presented, they were commonly reduced size or became conical or peg-shaped, but these appearances did not occur in non-syndromic hypodontia patients. In contrast to XLHED, among various positions of teeth of isolated tooth agenesis subjects, the anterior teeth seemed to be absence rather than the molars.

Focus on genotype, to date, there are 12 different mutations underlying non-syndromic hypodontia. Most of them were missense mutations, affecting the TNF. Whereas mutations caused XLHED could detected in any parts of the whole gene and could be any types of mutations including missense, insertion, deletion, and loss of the entire gene.