Verena RUPP

Systematic characterisation of consanguineous Pakistani families displaying various forms of alopecia

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Index

Abbreviations

AA	Alopecia areata
aa	Amino acid
AGA	Androgenetic alopecia
APCDD1	Adenomatosis polyposis coli down-regulated 1
ARWH	autosomal recessive woolly hair
BMP	Bone morphogenic protein
BMPR-IA	Bone morphogenic protein type IA receptor
CCN2	Connective tissue sheet growth factor
CD4+ / CD8+	Cluster of differentiation positive 4 / 8
CDH3	Cadherin-3
CDSN	Corneodesmosin
CMT2B1	Charcot-Marie-Tooth disease, axonal, type 2B1
CTS	Connective tissue sheet
DKK	Dickkopf
Dlx3	distal-less homeobox 3
DP	Dermal Papilla
DS	Dermal Sheet
Dsc	Desmocollin
Dsg4	Desmoglein-4
DSP	Desmoplakin
Eda-A1	Ectodysplasin- A1
EdaR	Ectodysplasin-A receptor
FGF	fibroblast growth factor
$G_{13/12}$	G-Protein 12/13

Gli-2	Glioma assoziated oncogene-2
HB-EGF	Heparin-binding EGF-like growth factor
Het	Heterozygous
HF	Hair follicle
HGF	Hepatic growth factor
HLA	human leukocyte antigen
Hom	homozygous
HR	Hairless
IGF-1	Insulin-like growth factor-1
IL-1	Interleukin 1
IRS	Inner Root Sheet
JmjC	Jumonji domain containing
JUP	junction plakoglobin
KIF	Keratin intermediate filaments
KRT / K	Keratin
LAH	Localized autosomal recessive hypotrichosis
Lef-1	Lymphoid enhancer-binding factor-1
Lgr4	Leucine-rich repeat containing G-protein coupled receptor 4
LIPH	Lipase member H
LMNA	Lamin A/C
LOD	Log-of-the-odds
LPA	Lysophosphatidic acid
Lpar6	Lysophosphatidic acid receptor 6
MAD	Matrix associated deacetylase bodies
MUT	Mutant
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells

ORS	Outer Root Sheet
p63	Tumor protein p63
P-cadherin	Placental cadherin
PDGF-A	Platelet– derived growth factor-A
PHNED	Pure hair and nail ectodermal dysplasia
PKP1	Plakophilin-1
Rho	Ras homolog
RORα	Retinoic acid receptor - related orphan receptor alpha
RPL21	Ribosomal protein L21
Shh	Sonic hedgehog
Smo	smoothened
SNP	Single- nucleotide-polymorphism
ST14	Suppression of tumorigenicity 14
TGF	Transforming growth factor
TNF-α	Tumor necrosis factor- α
U2HR	U2 upstream region of the 5’UTR of *HR*
VDR	Vitamin D receptor
VEGF	Vascular endothelial growth factor
WT	Wildtype
Wnt	Wingless-related MMTV integration site

1 Introduction

Curly, straight, long, or short; hair defines a persons' appearance. It is socially important in terms of attractiveness or expressing one's individuality (Cash, 2001). Sometimes it just needs a different hairstyle or haircut to change a person's whole appearance. Apart from the social factor, hair also has a protective function, e.g. against electromagnetic radiation, trauma, insect attacks; it is sensitive to environmental influences or thermal insulation. Furthermore, its cleansing function of the skin surface as well as the dispersion of sebum and pheromones, which act as environmental signals, are notable characteristics (Stenn and Paus, 2001).

1.1 The human hair follicle

The hair follicle itself is a mini organ consisting of eight concentric layers (Schneider et al., 2009; Oshima et al., 2001). Moreover, the actively growing anagen hair can be divided into a cycling lower portion, which is regenerated in every new hair cycle, and a more or less permanent upper part (Stenn and Paus, 2001; Schneider et al., 2009; Yoo et al., 2010). Figure 1.1 illustrates a schematic of the anagen hair follicle. The peripheral layer, the outer root sheet (ORS), and the inner root sheet (IRS) represent the epithelial core (Oshima et al., 2001). The border between the hair follicle and the dermis and subcutis is given by the cells of the ORS. It is composed of a single layer around the bulbar area and expands into multiple layers at the distal part of the follicle where glycogen containing cuboidal cells can be found (Stenn and Paus, 2001). Distal from the bulb, a protuberance, the bulge, is formed by the ORS cells, which contains the hair follicle stem cells (Schneider et al., 2009). Adjacent to the ORS is the companion layer, which is comprised of long, flat cells and forms a slippage plane enabling the cells of the IRS and hair shaft to move upwards against the ORS (Stenn and Paus, 2001; Alibardi, 2004b; Alibardi, 2004b; Alibardi, 2004a). Since it is connected to the Henle's layer but not to the ORS by desmosomes, it is supposed that the companion layer slides outwards together with the IRS. The Henle's and the Huxley's layer as well as the IRS cuticle constitute the IRS, respectively. In general, the companion layer and the ORS can be distinguished from the IRS, by the absence of trichohyalin in the cytoplasm, a marker of the IRS, and the medulla, the innermost part of the hair filament (Alibardi, 2004b; Orwin, 1971;

Steinert et al., 2003; Rothnagel and Roop, 1995). This highly charged α-helical protein mechanically strengthens the inner root sheet by cross-linking with the keratin intermediate filaments, KIF (Steinert et al., 2003; Lee et al., 1993). The IRS itself defines the morphology of the growing hair, ensures its proper anchoring in the follicle as well as the keratinization (also known as 'cornification'). It ends in the lower part of the isthmus, just beneath the sebum secreting sebaceous gland, marked by the finally fully cornified and degraded IRS cells. Other than his two neighbors, who are only single cellular layers, the Huxley's layer consists of three to four different cells and so called elongated 'Flugzellen', which are first seen in the upper region of the follicle bulb. These specialized cells are able to pass the already fully keratinized cells of the Henle's layer and interact with the cells of the ORS companion layer to provide nutrients for the not yet cornified Huxley's cells (Joshi RS, 2011). Furthermore, since the cuticle of the hair filament and the cuticle of the IRS are tightly interlocked through their scales, protection of the hair is given by the combined upwards growth (Joshi RS, 2011).

Beside the already mentioned hair cuticle, which forms the outermost part of the hair fibre, the cortex and the medulla are the remaining parts of the hair shaft that form together the mature hair filament. Their proper architecture is of considerable importance, since only an intact hair structure is able to guarantee the protection and absorption or reflection of light. In addition, it determines the structure and shape of the hair and thus influences the appearance of an individual. Keratinisation predominantly happens in the cortex, determining the strength of the fibre (Schlake, 2007). Out of 26 keratins specifically expressed in hair, 12 are only expressed in the cortex of scalp hair. Surprisingly, in the cortex of vellus hair a 13^{th} keratin, K37, can be detected, which also occurs in the medulla of sexual hair (Moll et al., 2008).

The innermost part of the hair fibre is made up by the medulla, a compartment with horizontally growing, vacuolated cells which occur in a hair type specific manner (Langbein et al., 2010). Interestingly, in murine as well as in some other mammalian hair but not in human, air spaces can be found in the medulla, which are supposed to influence the thermal insulation (Schneider et al., 2009; Langbein et al., 2010). For a long time it was believed that the medulla does not contain any keratins, but only recently Langbein et al (Langbein et al., 2010) confirmed the expression of 24 keratins by investigating beard hear medulla.

Hair pigmenting melanozytes are highly abundant in the cortex of the shaft, but occur

at minor rate also in the medulla and hardly in the cuticle. Although melanozytes can be found in other follicle compartments too, the ones actively producing melanin and influencing the shaft colour reside within the upper hair bulb matrix cells (Tobin et al., 1999). While the dark hair of the human scalp belongs to the highly pigmented terminal hair generating an effective sunscreen, the finer and lighter vellus hair covers the rest of the body, excluding non-hairy and pubic areas (Stenn and Paus, 2001; Van Neste and Tobin, 2004).

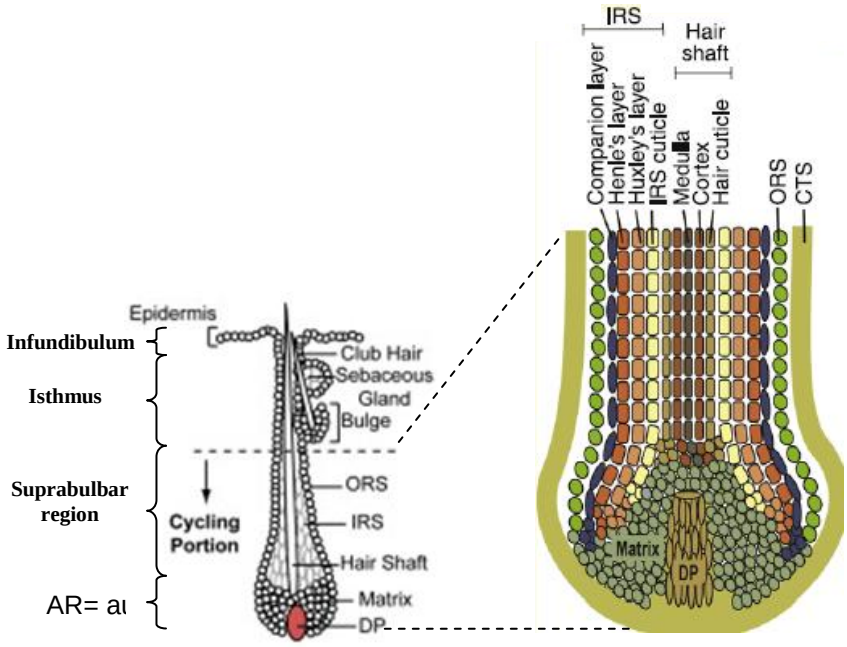

Fig. 1.1: **Schematic of the human hair follicle with highlighting the anagen-associated bulbar area**. Beneath the old bulge containing the dead and fully keratinized club hair, which is eventually about to be depleted during exogen phase, is suited at the border between cycling and permanent portion (Hsu et al., 2011; Trueb, 2010). The lower cycling unit includes the matrix and DP containing bulbar and suprabulbar region, while the permanent region is composed of the Isthmus- with the bulge and the sebasceous gland- and the Infundibulum consisting of the ORS and the hair canal. Not shown here is the arrector pili muscle, which inserts at the bugle region (Schneider et al., 2009; Hsu et al., 2011). ORS: outer root sheet, IRS: inner root sheet, DP: dermal papilla, CTS: connective tissue sheet or 'dermal sheet'. (Figures adapted from (Schneider et al., 2009) and (Hsu et al., 2011))

Located at the very bottom of the hair follicle, the highly proliferative matrix cells give rise to the different layers of the follicle mentioned above, except for the ORS, which is produced by locally coherently growing and apoptotic progenitors underlying a polyclonal origin. Legue and Nicolas (Legue and Nicolas, 2005) divided the matrix into three distinct layers, namely the stem cell containing germinative layer, connected with the dermal papilla, the progenitor layer, developing out of the self-

renewal stem cells of the germinative layer, and the final post-mitotic layer, resulting from symmetrically dividing transient progenitor cells. Thus they proved that the matrix is composed of different cells instead of equivalent multipotent ones.
In the very central part of the follicle the dermal papilla, consisting of specialized fibroblasts, can be found (Schneider et al., 2009; Ouji et al., 2012). Like the connective tissue sheet, a glassy double layer of loosely assembled collagen fibres and stromal cells that envelopes the follicle, the dermal papilla, derives from the mesoderm, while the remaining hair structures are of epithelial origin (Schneider et al., 2009; Rogers, 2004). The dermal papilla is important in terms of bulge stem cell activation in order to form a new follicle during the transition from the resting to the destruction phase (Hsu et al., 2011).

1.2 Signals involved in the hair follicle development and cycling

In 1992, Hardy M.H. (Hardy, 1992) wrote an overview about the 8 stages of the hair follicle development when its life cycle and the signals involved were still not very well understood. Following extensive studies in mice and cell culture there is a more detailed understanding of these processed today. Starting on embryonic day 14.5, an initial epithelial placode is formed through mesenchymal cell aggregation in the dermis of mice, giving the overlying keratinozytes of the epidermis a signal to start proliferating and thus induces an inwards growth forming the so called ‘plug’ (Schneider et al., 2009; Hardy, 1992; Lehman et al., 2009). These steps include stage 0-5 of the follicle formation (Lehman et al., 2009). However, signals involved in the primary dermal/epidermal interaction are still unknown, while for the placode and following plug formation several signaling have turned out to be crucial: Wnt/ β-catenin and their downstream effector Lef-1 (Lymphoid enhancer-binding factor-1), noggin, TGF-ß (Transforming growth factor-ß), (Lehman et al., 2009; Millar et al., 1999) Eda-A1 (Ectodysplasin- A1), its receptor EdaR (Ectodysplasin-A receptor) and their downstream effector NF-κB (Schneider et al., 2009; Botchkarev et al., 1999; Laurikkala et al., 2002; Pummila et al., 2007; Narhi et al., 2008; Kishimoto et al., 2000). Furthermore, Shh (sonic hedgehog), that is expressed in the epidermal placode with its downstream effectors Gli-2 (Glioma assoziated oncogene-2) and Smo (smoothened), the connective tissue sheet growth factor (CCN2), Lgr4 (Leucine-rich repeat containing G-protein coupled receptor 4), follistatin, FGF’s

(fibroblast growth factors) and their receptors, as well as P-cadherin do also play an essential role in this first hair follicle forming stage (Schneider et al., 2009; Lehman et al., 2009; Botchkarev et al., 1999; Pummila et al., 2007; Narhi et al., 2008; Kishimoto et al., 2000; Mohri et al., 2008; Leask et al., 2009).

BMPs (bone morphogenic protein) and Dickkopf (DKK) proteins are the antagonists of the signals inducing this primary follicle development (Kwack et al., 2012; Botchkarev et al., 2002; Krupnik et al., 1999). While, i.e. BMP2 inhibits ß-catenin/Lef-1 signaling in the hair placode, BMP4 is actively expressed in the mesenchymal cells, which have to form aggregates in order to enable the placode formation (Botchkarev et al., 2002).

Noggin is supposed to neutralize the BMP2/BMP4 interaction with its BMPR-IA receptor and thus activates the hair follicle morphogenesis in the embryo (Botchkarev et al., 1999). Interestingly, in *noggin* null mice the primary hair follicle development (known as 'tylotrich hair follicle'; it is first seen at embryonic day 14.5 in the murine dorsal skin forming a straight hair with a large bulb, and two sebaceous glands) seems to stay unaffected by the missing noggin expression, while the induction of the secondary hair follicle formation (non-tylotrich HF with shorter awl and zigzag formed hair filaments growing between embryonic day 16.5 until shortly after birth) was proven to require the down regulation of the BMP signaling by the action of its antagonist noggin (Botchkarev et al., 2002). In contrast, Laurikkala et al (Laurikkala et al., 2002) reported, that mice lacking the *Eda* (*Ecdodysplasin-A*) gene were unable to form primary hair follicles. These findings suggested that noggin is required for the secondary hair follicle formation, while Eda regulates the primary hair follicle morphogenesis (Botchkarev et al., 2002). Another important molecule in the hair follicle development is DKK1, which is supposed to antagonize WNT signaling since it was detected to inhibit placode development when over expressed in mice. Moreover, DKK1 plays an important role in the postnatal hair follicle cycling by promoting the inhibition of WNT/ß-catenin signaling and inducing the apoptosis of keratinozytes during the transition from anagen to catagen (Narhi et al., 2008; Kwack et al., 2012).

During the first 5 stages also the dermal papilla, for which Shh, Gli2 and PDGF-A (platelet –derived growth factor-A) signaling are pivotal, is developed. This happens when the mesenchymal cells, which have aggregated in the beginning, are being surrounded by epithelial cells from the initial hair plug and later become the above

mentioned highly differentiating matrix cells (Hardy, 1992; Lehman et al., 2009). Dependent on how many cells were involved in this formation, the thickness and size of the resulting hair filament and follicle are being defined during this stage. Finally, the remaining hair structures, e.g. IRS and hair filament are developed after another dermal signal has been emitted from the dermal papilla to the adjacent matrix cells, making them grow upwards and differentiate into the specific layers (Hardy, 1992). With this mechanism around 100.000 hair follicles and fibers are developed on the scalp and 5 million on the entire body (Paus and Foitzik, 2004).

After birth the postnatal hair follicle cycling occurs in an asynchronous manner in human with every follicle underlying a unique cycling pattern consisting of an active anagen phase, the shortening catagen phase, the resting telogen period, and the final hair dislodging exogen phase (Milner et al., 2002)or teloptosis (Schneider et al., 2009; Hardy, 1992; Milner et al., 2002; Pierard-Franchimont and Pierard, 2001; Lavker et al., 1993; Kunz et al., 2009). A fifth phase was suggested by Rebora and Guarrera in 2002 (Rebora and Guarrera, 2002), which they called ‘kenogen’, describing the phenomenon when the hair follicle remains empty after the exogen shedding, e.g. when suffering from androgenetic alopecia. Only recently the shift from telogen to anagen has been suggested to be named ‘neogen’ (Bernard, 2012). However, active hair growth in humans was observed to last up to 6 years, followed by a short catagen (2 weeks) and a final resting (telogen) phase lasting 2-3 months before shedding occurs eventually (Trueb, 2010; Lavker et al., 1993; Kunz et al., 2009). For up to five months more than 80% of all hair cycles rest after the shedding in the kenogen phase before a new hair is formed in anagen (Bernard, 2012).
The signal for a new hair is already transmitted during the catagen to telogen transition. After the follicular matrix cells have undergone apoptosis during catagen, the old hair is pushed upwards and its dermal papilla gets in touch with bulge stem cells that start to differentiate and migrate downwards to create the ORS and later the hair germ. Meanwhile, the old club hair still remains in the hair canal until it is shed in exogen (Hsu et al., 2011; Paus and Foitzik, 2004).

Several signals, e.g. WNTs, BMPs, DKKs or Shh, which have already been mentioned to be crucial during the hair follicle morphogenesis, also play a striking role in the postnatal cycle. To initiate anagen, for instance, molecules which had

been highly expressed during early telogen, such as BMP2, BMP4, DKK1, FGF18 and Sfrp4, are down regulated during the refractory telogen-to-competent anagen switch during which WNT/ß-catenin, TGF-ß, Fgf-7/10 and noggin are being highly expressed (Rebora and Guarrera, 2002; Kimura-Ueki et al., 2012; Plikus et al., 2009; Plikus et al., 2008). By the time of anagen to catagen transition high levels of FGF-5, TGF1, IL-1, several neurotrophins, or DKK1 have been observed, which are being counteracted by hepatic growth factor (HGF), vascular endothelial growth factor (VEGF), and insulin-like growth factor-1 (IGF-1) and controlled by the interaction between the members of the TNF-α signaling pathway and Keratin 17 (Schneider et al., 2009).

Mutations in several of those cell cycle specific signaling genes or proteins required for the hair follicle morphogenesis are known to result in abnormal hair growth or morphology. If overexpressed, Noggin, for instance, leads to an enormous loss of hair in transgenic mice, whereas extended hair growth was found in FGF-5 knockout mice (Kulessa et al., 2000; Ahmad et al., 1999; Ahmad et al., 1998a). Another member of the fibroblast growth factor family, FGF-7, leads to greasy and matt hair if disrupted (Ahmad et al., 1998a).

1.3 The hair and its diseases

However, there are numerous diseases affecting the hair growth and cases in which stress, nutrient deficiency, medication therapy-dependent abnormal hair growth, or loss occurred have been reported. Although gain or loss of hair is not life threatening, it appears to be a burden for those affected. Especially the reaction from society on their hair problems ends in even more severe psychological distress, frequently leading to depression (Cash, 2001; Hadshiew et al., 2004; Lee et al., 2011). Hardshiew et al (Hadshiew et al., 2004) summarized all the negative effects of alopecia on the patient personal perception, e.g. including reduction in their self-confidence, powerlessness, sadness, depression, embarrassment, reduced social acceptance, or sense of inadequacy.

Normally, up to 100 hair filaments can be shed per day, but hair loss turns clinical if only around 50% of the scalp hair remains present (Trueb, 2010; Courtois et al., 1996; Zhao et al., 2008). Probably the most common type of alopecia is the androgenetic hair loss (androgenetic alopecia, AGA), not only affecting men but also

women. By the age of 60, 2/3rd of all men exhibit AGA, with a higher prevalence in Caucasians (Cash, 2001). For this type of hair loss a sensitivity of the hair follicles against androgen that reaches the follicle through the blood vessels is responsible, and thus inducing the release of inhibitory signal molecules such as DKK1, IGF-1 (insulin-like growth factor 1) or TGFß-1 and 2 (Uchiyama et al., 2012). A more severe type of hair loss prevalent in 0,2% of people worldwide (Gilhar et al., 2012) is the autoimmune disease alopecia areata (AA), with the infiltrating lymphocytes of the immune system attacking the hair follicle (Gilhar et al., 2012; Petukhova et al., 2011). Initially, patches of hair loss can be observed on the scalp or in the area of the beard (AA simplex), which either remain like this or spread further and affect the entire scalp hair (alopecia areata totalis) or even the entire body hair (alopecia areata universalis) (Petukhova et al., 2011; Petukhova et al., 2010). Although the pathomechanism is similar in all three types, only a few patients suffering from mild forms of AA respond to a topical or intralesional steroids therapy (Petukhova et al., 2011; Ito, 2012; Ito, 2012). However, the inheritance mechanism and genetic background of this disease still remains more or less elusive, but it is suspected to be a polygenetic disease differing in terms of penetrance (Ahmad et al., 1998a; Petukhova et al., 2010).

A recent survey from Bernard et al (Bernard et al., 2011) among lung cancer patients revealed, that 93% of 135 patients were willing to pay more for a chemotherapy drug if it decreased the risk for chemotherapy-induced alopecia from 40% to 5%. While for stress, medication, androgenetic or nutrition-level dependent as well as for chemotherapy-induced alopecia pharmacological interventions and therapies are already available, for some more severe types of hair diseases, like alopecia totalis, alopecia universalis, or congenital alopecia, suitable medical treatments still have to be developed. (Cash, 2001; Yoo et al., 2010; Zhao et al., 2008; Uchiyama et al., 2012; Ito, 2012; Yeager and Olsen, 2011)

In the last 15 years a multitude of new genes involved in congenital bolding and abnormal hair growth patterns in human has been discovered. Around 50 different types of syndromic and non-syndromic congenital hair loss disorders have been reported so far, including atrichia with papular lesions, wooly hair, Marie Unna hypotrichosis, autosomal recessive hypotrichosis, Naxos disease, Carvajal syndrome, as well as the keratin disorders monilethrix, ectodermal dysplasia,

autosomal dominant hypotrichosis and wooly hair. In fact, these diseases occur less frequently compared to the AA mentioned above, but in order to understand the mechanism of hair growth and the follicle development, it is important to unravel the genes responsible for these congenital hair disorders (Shimomura, 2012). Table 1.1 summarizes the most important congenital hair disorders and their corresponding genes found so far. Non-syndromic forms of hair loss, like those covered in this work, indicate congenital alopecia, autosomal hereditary recessive, as well as dominant hypotrichosis/ wooly hair, generalized hypotrichosis simplex, Marie Unna hypotrichosis, and autosomal dominant and recessive Monilethrix (Shimomura, 2012).

Table 1.1: Most important congenital hair diseases and their associated genes (created according to the paper of Shimomura Y., 2012 (Shimomura, 2012))

Disease	Inheritance pattern	Phenotype	Genes	Reference
Congenital alopecia/ Universal congenital alopecia	AR	Complete loss of hair after the postnatal ritual shedding on the entire body	HR	(Ahmad et al., 1998a)
Atrichia with papular lesions	AR	Complete loss of hair only scalp or entire body hair, sparse eyebrows and eyelashes, keratin filled cysts on skin. Hereditary 1,25-dihydroxyvitamin D-Resistant Rickets in patients with VDR mutation	HR, VDR	(Ahmad et al., 1998b; Miller et al., 2001)
Autosomal recessive hypotrichosis/ wooly hair	AR	Fine and extremely kinky hair / Sparse scalp hair, remaining body hair appears normal in most patients with LIPH and LPAR6 mutations but is sparse in those with DSG4 mutations	LIPH, LPAR6 (P2RY5), DSG4	(Shimomura et al., 2008; Kazantseva et al., 2006; Kljuic et al., 2003; Pasternack et al., 2008)
autosomal dominant wooly hair/ hypotrichosis	AD	tightly curled hair / normal to sparse hair	KRT74, K71	(Shimomura et al., 2010; Fujimoto et al., 2012)
Monilethrix	AR/AD	Fragile and sparse hair , nail deformations; in patients with DSG4 mutations keratosis pilaris and erythematous follicular papules on the scalp can be detected	DSG4 (AR), KRT81, KRT83, KRT86 (AD)	(Shimomura, 2012; Kljuic et al., 2003)
autosmal recessive ichthyosis with hypotrichosis	AR	Sparse, curly, brittle hair; diffuse body skin scaling without affecting the face	ST14	(Basel-Vanagaite et al., 2007)
hypotrichosis and recurrent skin vesicles	AR	Fragile, sparse hair and mucosal vesicles on scalp and body skin	DSC3	(Ayub et al., 2009)
hypotrichosis with juvenil macular dystrophy	AR	Postnatal alopecia with sparsely re-growing short hair in adolescence, peripheral retinal dystrophy	CDH3 (P-cadherin)	(Sprecher et al., 2001)
ectodermal dysplasia/skin fragility syndrome	AR	Short and sparse hair, skin blistering with accompanied scaling, thick nails with dystrophy	PKP1	(McGrath et al., 1997)
Naxos disease	AR	Arrythmogenic right ventricular cardiomyopathy , palmoplantar keratoderma, wooly hair	JUP, DSC2	(McKoy et al., 2000; Simpson et al., 2009)
Carvajal disease	AR	Left ventricular cardiomyopathy, palmoplantar keratoderma and wooly hair	DSP	(Norgett et al., 2000)
pure hair and nail ectodermal dysplasia (PHNED)	AR/AD	Sparse hair, nail dystrophy, short an fragile hair	KRT85 (AR), no gene for AD PHNED	(Shimomura, 2012; Naeem et al., 2006)
ectodermal dysplasia, ecrodactyly and macular dystrophy	AR/AD	Ecrodactyly, sparse and kinky hair, weak eyesight	CDH3 (AR), p63 (AD)	(Sprecher et al., 2001; Shimomura et al., 2008)
hereditary hypotrichosis simplex of the scalp	AD	Continuously hair loss starting in the first decade of life. Either only scalp or entire body hair affected.	CDSN	(Shimomura, 2012; Levy-Nissenbaum et al., 2003)
generalized hypotrichosis simplex	AD	Sparse and thin scalp and body hair	APCDD1, RPL21	(Zhou et al., 2011; Shimomura et al., 2010)
Marie Unna hypotrichosis	AD	Sparse scalp and facial hair at birth. Patients develop coarse and wiry hair in childhood and alopecia in puberty	5'UTR of HR	(Wen et al., 2009)

AR= autosomal recessive, AD= autosomal dominant

1.4 The impact of consanguinity on the identification of disease genes

During the work on this thesis only consanguineous Pakistani alopecia families with recessive inheritance pattern were investigated. Although the rate of inbred marriages has declined in the last decades, still several countries and minorities are practicing this tradition; not only in Asian, Arab, and African countries, but also on the American subcontinent, where the Amish people are well known to suffer more often from rare recessive diseases compared to the non-consanguineous population (Payne et al., 2011). In Pakistan, for instance, up to 49% of all marriages are between relatives (Jaber et al., 1998). In general, consanguineous marriages increase the risk of developing recessive diseases due to the decrease in genetic heterogeneity (Jaber et al., 1998; Stoltenberg et al., 1999; Petukhova et al., 2009). On this basis, for geneticists such families provide an ideal basis to unravel the underlying genetic cause and thus the molecular mechanisms of rare recessive inherited diseases. Consanguineous couples often share the same allelic segments from a common ancestor which can be passed on to the offspring twice. These so called 'autozygous' blocks can be detected by homozygosity mapping, a technique used for revealing the homozygous chromosomal region in the affected children which most likely carries the recessive disease gene. As a result, the finding of such genes in inbreed families makes the mutational screening of patients who descend from non-consanguineous families and do not carry autozygous segments much easier, as it would be rather challenging without knowing any genes to look for (Jaber et al., 1998; Alkuraya, 2010).

Lately, genome wide autozygosity mapping has been conducted with single-nucleotide-polymorphism (SNP) arrays, a fast and cost-efficient method to detect homozygous regions in the genome (Alkuraya, 2010). This approach is based on the assumption that SNPs within the region where the identical-by-descent mutation is suspected to be located also have to be homozygous (Hildebrandt et al., 2009) . A SNP is defined as a single base pair variation that can be found in more than 1% of all genomes of a certain population and can be thus used as a marker for segregation analysis (Brookes, 1999). To date, 178.140.935 SNPs have been submitted to the dbSNP database on NCBI. 52.327.221 of these are reference SNPs of which 41.740.143 are validated and 21.247.880 SNPs are reported to reside in a gene (Sherry et al., 2001). In principle, for the SNP array the DNA of an individual is

first amplified with universal primers, next the acquired fragments are fluorescence labeled on their ends and finally they are hybridized to a microarray chip containing oligonucleotide probes with all possible SNPs for a given position[1] (Sudbery Peter, 2009). For each SNP only 2 genotypes, A or B, are possible, giving either an A/A, B/B (homozygous for A or B), or an AB (heterozygous for A and B) call (Lin et al., 2004). If a low density SNP array was used (like a 10.000 SNPs carrying 10K array), further fine mapping of the filtered regions can be conducted by more informative microsatellite marker analysis to define the general borders of the homozygous region in all affected individuals of the family (Petukhova et al., 2009).

To confirm the data achieved from autozygosity mapping, the linkage between the microsatellite markers and the disease locus, that also represents a marker, can be analyzed by LOD (log-of-the-odds) score calculation. This gives information about the likelihood that two inherited loci are linked due to genetic linkage or the two loci are linked by chance. A LOD score of >3 is said to be significant for a linkage at a given recombination fraction. A recombination fraction of $\theta=0$ indicates the odds of a linkage, while a recombination fraction of 0,5 means that the loci are not linked. For this calculation at least two markers are required (two point analysis), but multiple loci can be also used (multipoint analysis)[2] (Sudbery Peter, 2009).

The aim of this thesis was to unravel the underlying genetic cause of the autosomal recessive inherited hair disorders in five consanguineous Pakistani families by a positional cloning strategy (or linkage mapping (Kwon and Goate, 2000)). For this, one or two affected individuals from each family- dependent on their familiar relationship- were sent to the “Centre for medical research” in Graz for SNP Array analysis. The received SNP Array data were screened for homozygous or compound heterozygous regions to link the disease to a certain locus. The segregation of the locus in the family was later determined via polymorphic microsatellite marker analysis. Candidate disease genes, identified by genetic database and literature research, that were lying within this locus were sequenced. After revealing a

[1] Sudbery, Peter/ Sudbery Ian (2009). p.130f

[2] Sudbery, Peter/ Sudbery Ian (2009). p.61-64, 85

mutation, the familiar segregation of the detected mutations was also validated by sequencing.

However, since family AP2 revealed neither a known homozygous nor a known compound heterozygous region for alopecia, hypotrichosis, or woolly hair, Next Generation Sequencing of the DNA of one or two affected individuals would be a possible next step.

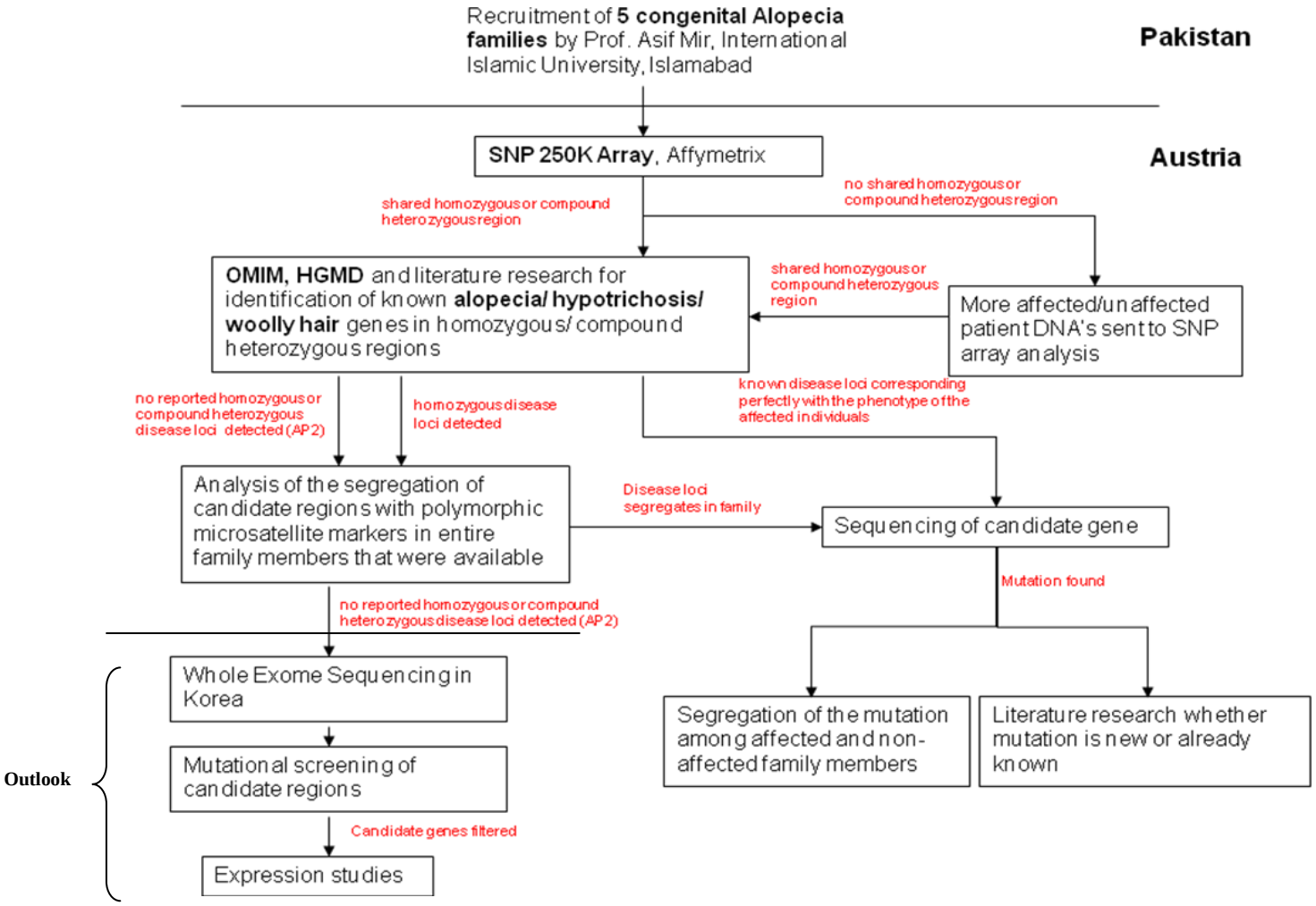

Fig.1.2: Positional cloning workflow

2 Material and Methods

2.1 Families

Five Pakistani Families affected by autosomal recessive inherited hair disorders were recruited from Dr. Asif Mir from the International Islamic University in Islamabad. After obtaining informed consent including the use of data and pictures for publication, their blood was collected and the DNA was sent to Graz for a mutational screening in March 2011. We received genomic DNA of numerous family member (affected and unaffected), pictures and family pedigrees. Pedigrees were redrawn for further applications with Cyrillic2.1 software (available from CyrillicSoftware, The Bagnold Wing, UK).

Due to missing hair samples, microscopic investigations of the hair morphology and the hair follicle were not possible.

2.1.1 AP1

From this family no clinical information beside pictures of the two affected male individuals AP1-2 and AP1-8 and a pedigree were available. The amount of hair on the scalp and eyebrows seems normal. The hair structure, however, appears quite curly and very thin, which resembles the typical phenotype for the so called ‘woolly hair’. Individual AP1-2 has shaved hair, which complicates the clinical diagnostic. From the 7 affected individuals of this family, only 6 (AP1-1, AP1-4, AP1-2, AP1-12, AP1-7 and AP1-8) were available for mutational screening, as well as 6 non-affected family members (AP1-5, AP1-6, AP1-9, AP1-10 and AP1-11). As demonstrated in the pedigree of the family (Fig. 2.2A), both branches are consanguineous.

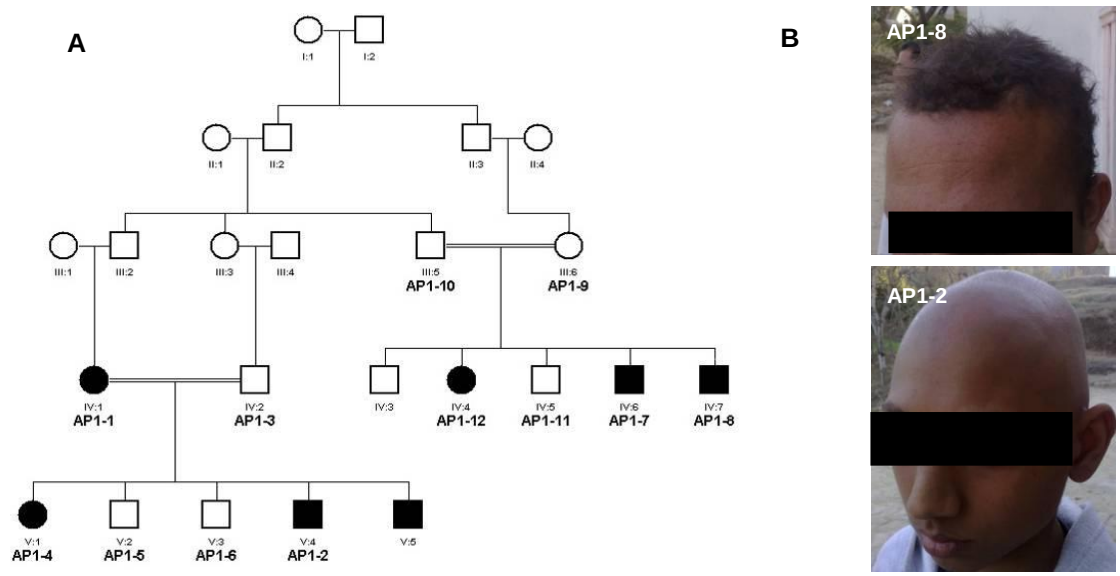

Fig. 2.2: Pedigree and pictures of family AP1. A. DNA was available from all AP named individuals (AP1-1 to AP1-12). Black symbols indicate affected individuals; white ones non-affected family members. Circles represent females, squares represent males. Double horizontal lines between couples represent consanguineous mating. **B**. Affected individuals AP1-8 and AP1-2. The disease phenotype can only be determined through individual AP1-8.

2.1.2 AP2

For this family it is hard to make a statement about consanguinity, since the pedigree contains only three generations. Hair loss first appeared in the third generation after marriages among members of the second generation in the pedigree. All four affected individuals of the AP2 pedigree (AP2-1, AP2-2, AP2-3 and AP2-4) have sparse to no hair on the scalp, with a little more hair remaining on the back of the head. The hair itself has no curly appearance. Interestingly, this kind of hair loss obviously does neither really resemble any alopecia nor hypotrichosis forms reported so far. Eyebrows, eyelashes, the remaining body hair, as well as the patients' skin are reported to be normal. The two affected girls have unremarkable nails; those of the men appear hard and dark coloured. Even though the nails of the girls are partly orange coloured we expect this to result rather from henna painting, a common practice among Muslim women in Islamic countries (Polat et al., 2009), than from a genetic disorder, but we still do not have any confirmation for this assumption.

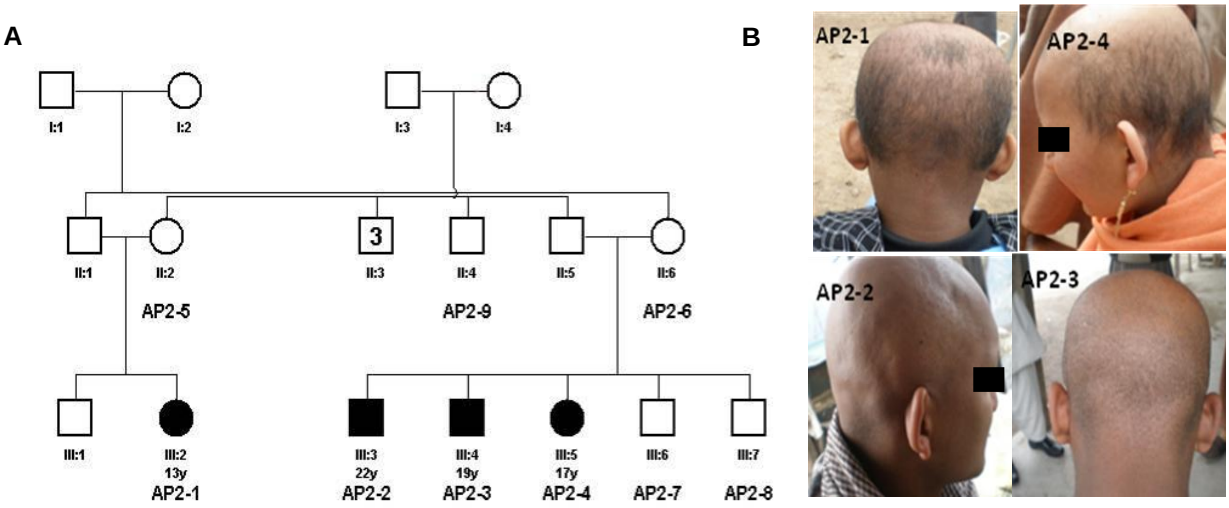

Fig. 2.3: Pedigree and pictures of Family AP2. A. DNA was available from all 'AP' named individuals (AP2-1 to AP2-9). Black symbols indicate affected individuals; white ones non-affected family members. Circles represent females, squares represent males. Symbols with integrated numbers indicate the amount of siblings of the same gender. The age of the affected individuals is indicated under the symbols. **B**. Phenotypic appearance of patients AP2-1, AP2-2, AP2-4 and AP2-3.

The hyperkeratinisation found on the patients' heels, which is combined with a roughness of the skin around this region, is also remarkable. On the back of the feet brownish spots can be detected.

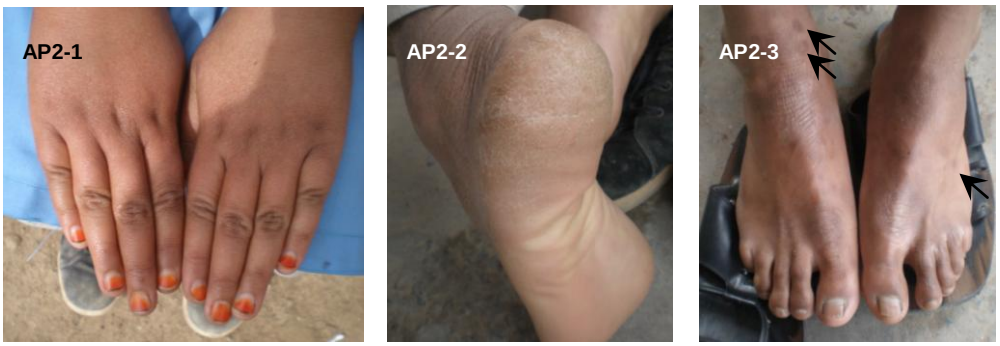

Fig. 2.4: Hands and feet of AP2 patients. The finger nails of the girls, AP2-1 and AP2-4, have an orange colour, which is suspected to be caused by Henna. A picture of the left sole of patient AP2-2 was picked to display the hyperkeratotic character of the skin on the heels of the patients. Black arrows indicate brownish spots on the back of the feet of patient AP2-3.

Moreover, the ankles were reported to bleed sometimes and the palms of the hands appear slightly thicker than normal. According to the information available, these latter abnormalities can only be reported from the siblings Ap2-2, Ap2-3 and AP2-4,

since no pictures of hands and feet were available from their cousin AP2-1. Although we did request information on this particular phenotypic appearance among the rest of the family members, we have not received any yet. As in family AP1, the diagnostic of the hair phenotype among the affected men is difficult due to their shaved scalps. Nevertheless, there is some growing hair visible on the pictures, but especially for individual AP2-3 it is hard to make a statement about his hair loss only by the pictures available. However, compared to the three other patients, he seems to have far more hair on the scalp, giving rise to doubts that he is also affected from alopecia.

Phenotypic abnormalities which are assumed not to stand in any relationship with the hair loss are the incomplete syndactyly between the 3rd and 4th finger on the left hand of individual AP2-4, as well as between the 2nd and 3rd toe of the left foot of individuals AP2-4 and AP2-2. The latter patient is also affected by a cleft lip and palate and a single tansverse palmar crease.

2.1.3 AP3

In this consanguineous family hair loss has occurred first among the 5th generation of the pedigree. The hair of the affected is sparse, slightly curled and appears untypically thin. Skin, nails, eyebrows and teeth are normal and also no other abnormalities were reported. Interestingly, the 12 year old girl, AP3-1, does have slightly more and longer hair compared to her younger brothers. The 9 year old brother, AP3-2, has the shortest and the least hair from all three affected, while the 1 year old patient AP3-3 has the most curly hair structure and is still suffering from rapid loss of hair.
DNA was only available from five of the 22 family members listed on the pedigree.

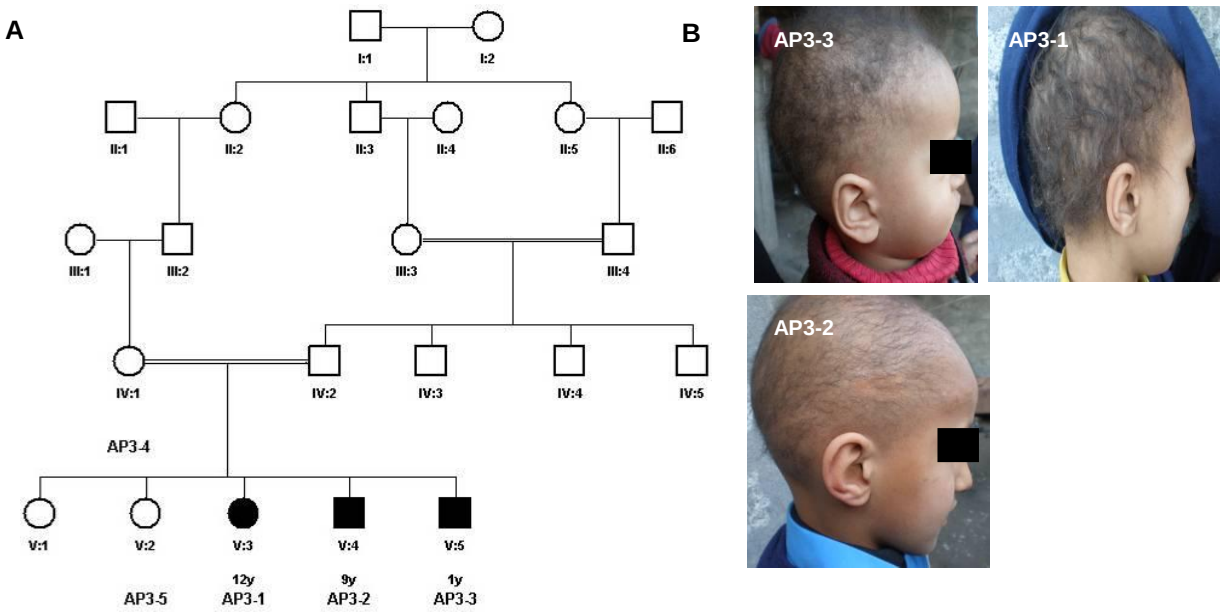

Fig. 2.4: Pedigree and pictures of Family AP3. A. DNA was available from all 'AP' named individuals (AP3-1 to AP3-5). Black symbols indicate affected individuals; white ones non-affected family members. Circles represent females, squares represent males. The age of the affected individuals is indicated under the symbols. **B**. Phenotypic appearance of patients AP3-1, AP3-2 and AP3-3

2.1.4 AP4

Affected individuals in this family have lost their entire body hair, with still countable eyelashes and eyebrows remaining on the 11 months and 3-4 year old brothers. Moreover, on patient AP4-3s' head numerous white pigmented spots are visible. The phenotypic appearance of the skin o of the two brother's heads resembles goose bumps. Although the disease could be due to a novel autosomal dominant mutation in individual AP4-4, the highly consanguineous family leads rather to the assumption that the disease causing mutation has to be an autosomal recessive one.

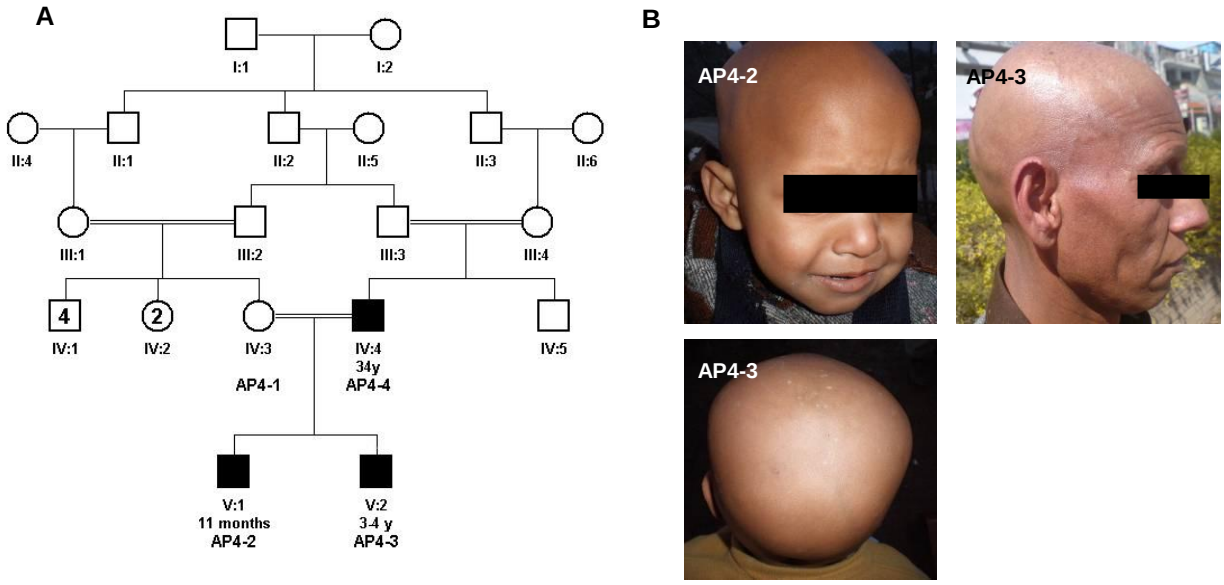

Fig. 2.5: Pedigree and pictures of Family AP4. A. DNA was available from all 'AP' named individuals (AP4-1 to AP4-4). Black symbols indicate affected individuals; white ones non-affected family members. Circles represent females, squares represent males. Symbols with integrated numbers indicate the amount of siblings of the same gender. The age of the affected individuals is indicated under the symbols. **B**. Phenotypic appearance of patients AP4-2, AP4-3 and AP4-4.

2.1.5 AP5

Like the affected members in family AP4, the patients of this consanguineous family display an autosomal recessive inheritance pattern and the same phenotypic appearance. Again we were just offered a pedigree and some pictures of the affected, but no clinical information or age were available. Of the 49 family members in the pedigree eight are affected from alopecia, with no hair on the entire scalp. The pictures reveal papular lesions all over the skin of the face of individuals AP5-2 and AP5-3. Among the very young affected, countable eyebrows, eye lashes, and beard hair are visible, while the elderly do not have any hair at all.

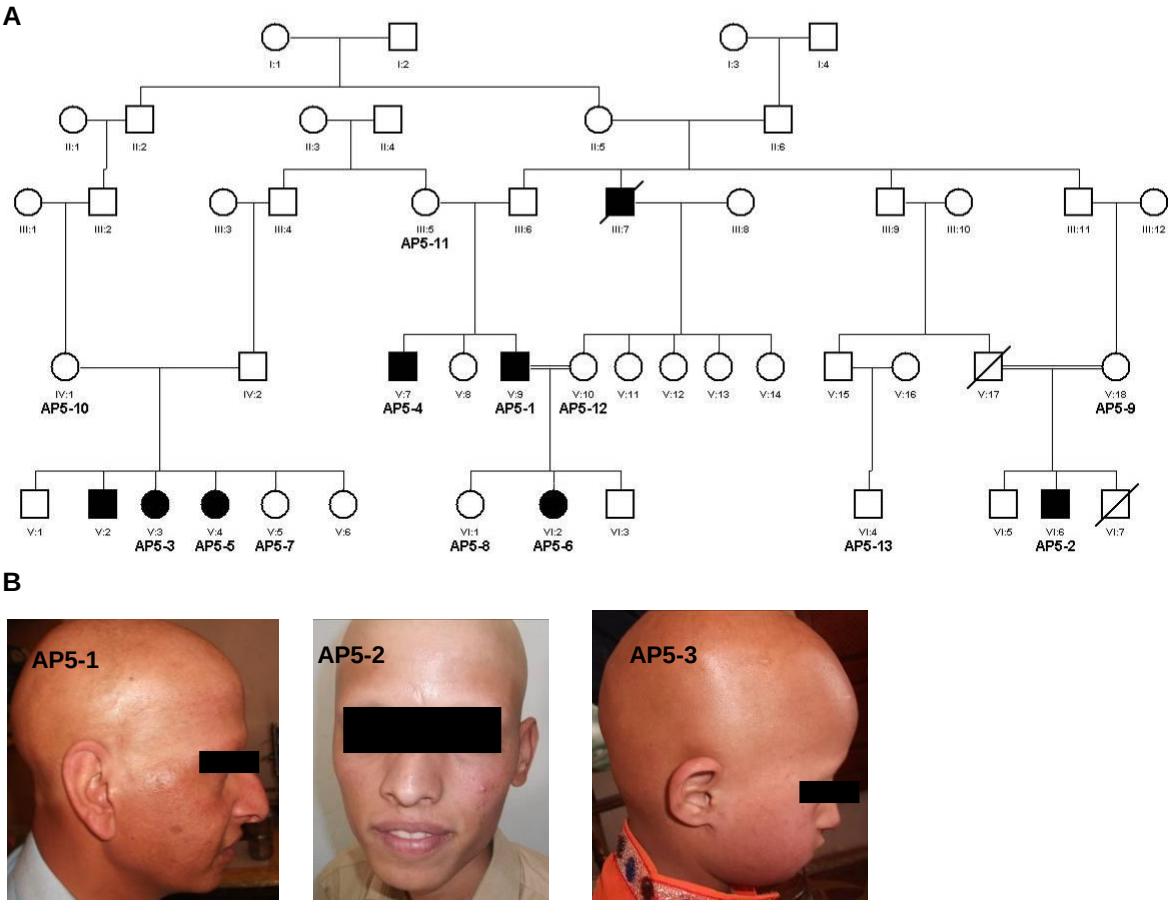

Fig. 2.6: Pedigree and pictures of Family AP5. A. DNA was available from all 'AP' named individuals (AP5-1 to AP5-12). Black symbols indicate affected individuals; white ones non-affected family members. Circles represent females, squares represent males. Symbols with integrated numbers indicate the amount of siblings of the same gender. The age of the affected individuals is indicated under the symbols. **B**. Phenotypic appearance of patients AP5-1, AP5-2 and AP5-3.

2.2 Homozygosity mapping

2.2.1 Affymetrix GeneChip Mapping 250K NSP

The Array was conducted according to the Affymetrix protocol at the "Center for medical research" of the Medical University of Graz.

Other than recommended only *NSP I* instead of both, *NSP I* and *STY I,* was used for the enzyme digestion of the DNA (250ng). Primer-specific adaptors were ligated to the generated *NSPI* restriction fragments for the following amplification of the fragments. After the PCR, the products were first fragmented through vortexing, denaturated, end-labelled and the probes were finally hybridised onto the array chip. The raw data were acquired from the "GeneChip® Operating Software" from

Affymetrix. dChip software (Lin et al., 2004) (available from http://biosun1.harvard.edu/complab/dchip/) was used to analyze the SNP 250K data in order to identify common homozygous regions among affected family members. The following settings were used:

Table 2.1: dChip analysis settings.

Open Group:	
File type	CEL
Suffix of TXT/CHP call files	.txt
CDF file	Mapping250K_Nsp.cdf
Array type	500K
Gene or SNP info file	Mapping250K_Nsp snp info.txt
Chromosome:	
Genome information file	Mapping250k_Nsp_genome_info_hg18.txt
Cytoband file	cytoBand hg18 sorted.txt
Gene or SNP list file	All genes or SNPs
Chromosome	0
Analysis method	LOH
Organism	Human

All required files are available for download on the dChip homepage (http://biosun1.harvard.edu/complab/dchip/) and have to be present in one single folder together with the array data for the analysis.

The first heterozygous SNP adjacent to the common homozygous regions was documented for filtering the loci. For the compound heterozygous loci, the first adjacent SNPs that aberrated from the haplotypes of the other patients were used for defining the borders of the loci.

2.2.1.1 Copy number variation analysis for family AP2

For a copy number variation analysis, 24 randomly chosen SNP array data were used to normalize the AP2 family member data. A list indicating the array name, the

sample name and the ploidy was generated in Microsoft Excel but saved as a .txt file. Gender and the numeric contamination columns were neglected. In general the same set-up as described in table 2.1 was used. However, under Analysis/open group also 'Analysis/Normalize & MBEI afterwards' (MBEI=Model based expression) and under 'Options/Background subtraction' the 'Mismatch probe (PM/MM difference)' were selected.

2.2.2 Microsatellite Marker Analysis

For Linkage and Segregation analysis of the homozygous and compound heterozygous regions following dinucleotide markers were selected on UCSC Browser (GRCh37/hg19) (http://genome.ucsc.edu/) (Kent et al., 2002): D1S303, D1S506, D13S164 and D13S1307. PCR Primers for these markers were designed with Primer3 software (Rozen and Skaletsky, 2000) and subsequently ordered from Microsynth AG (Swiss). Markers from the ABI Prism® Linkage Mapping Set (Applied Biosystems) were used for fine-mapping the regions of interest.

Table 2.2: Polymorphic microsatellite markers used for the segregation analysis.

Family	Region of interest	Chromosome	Marker	Position	Dye	ASR
AP1	42,065,578-61,276,109	13	D13S263	42,980,920	NED	146-174
		13	D13S153	48,790,734	NED	89-121
AP2	154,625,632-158,095,159	1	D1S303	155,637,498	FAM	181-191
		1	D1S506	156,845,396	NED	123-141
AP3	38,205,546-74,369,804	13	D13S218	39,032,231	FAM	141-153
		13	D13S263	42,980,920	NED	146-174
		13	D13S153	48,790,734	NED	89-121
		13	D13S156	74,657,425	VIC	276-296
AP5	21,971,932-21,988,565	8	D8S549	15,649,577	VIC	72-82
		8	D8S258	20,367,167	VIC	141-155
		8	D8S1771	25,441,114	NED	343-367

ASR= Allele size range

Amplification of the microsatellite markers was conducted in a 15µl reaction containing:

9µl True Allele® PCR Premix (Applied Biosystems)
1µl Primer Mix (5µm/each Primer)
1µl DNA (~50ng/µl)
4µl RNase free PCR grade H_2O (Qiagen).

Following cycling conditions were used:

Table 2.3: Cycling conditions for microsatellite marker amplification

1	95°C	12min
2	94°C	15ssec
3	55°C	15sec
4	72°C	30sec
Go to step 2→ **10x**		
5	89°C	15sec
6	55°C	15sec
7	72°C	30sec
Go to step 5→ **20x**		
8	72°C	10min
9	6°C	∞

0,4µl of each PCR product were denaturated in a 10µl Premix of Hi-Di Formamide™ with Gene Ruler ™ 500 –LIZ™ Size Standard (both from Applied Biosystems) at 95°C for 1,5 minutes and kept on ice for 5 minutes before the raw data were generated on the ABI 3130*xl* Genetic Analyzer. The length of the microsatellite markers was finally analyzed with the PeakScanner software v1.0 (Applied Biosystems).

In case of segregation of the candidate loci, the 'Online Medelian Inheritance in Man' database, OMIM ((McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University (Baltimore, MD), 2012) http://www.ncbi.nlm.nih.gov/omim), PubMed (http://www.ncbi.nlm.nih.gov/pubmed) and HGMD ((Stenson et al., 2003) http://www.hgmd.org/) were screened for genes lying within this region, which were reported to cause alopecia universalis, hypotrichosis, or woolly hair.

2.3 LOD score calculation

LOD scores for the homozygous region on chromosome 1 in family AP2 were calculated using the easyLINKAGE Plus v5.08 software (Hoffmann and Lindner, 2005). For each marker a specific marker file (.abi) including marker name, person

ID's, and allele size ranges was generated (see Fig.2.8). The 'Lane' numbers were chosen randomly. The pedigree structure file (.pro) included the family ID, person ID, father ID, mother ID, sex (1=male, 2=female, 0=unknown), the affection status (1=affected, 2=unaffected, 0=unknown), and the availability of the DNA (2=available, 0=not available), respectively (see Fig. 2.7). For the analysis, the Marshfield STRP map Human v35.1 was chosen. The LOD score was calculated by conducting a SuperLink v1.6 two-point parametric analysis as well as a GeneHunter multipoint linkage analysis. Both analyses were conducted by determining the recombination increment step to be 0.05 and the disease allele frequency was assumed to be 0,001 with a 99% penetrance.

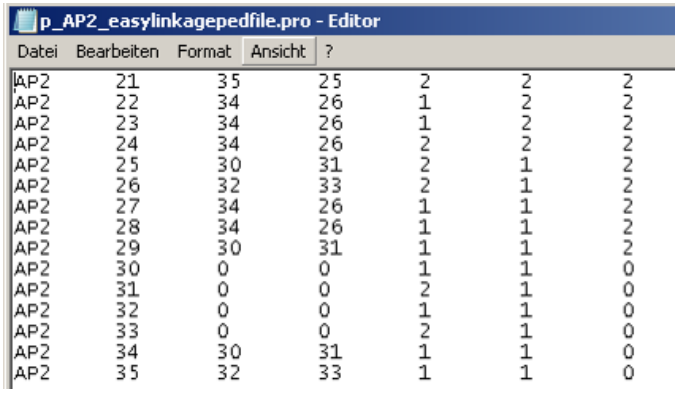

Fig.2.7: Pedigree file of family AP2 for the easyLinkage LOD score calculation. First column indicates the family ID, AP2. Column 2 indicates the person ID (21=AP2-1, 22=AP2-2, 23=AP2-3, 24=AP2-4, 25=AP2-5, 26=AP2-6, 27=AP2-7, 28=AP2-8, 29=AP2-9, 30=I:3, 31=I:4, 32=I:1, 33=I:2, 34=II:5, 35=II:1), column 3 indicates the father ID, and column 4 the mother ID (0=father/mother unknown), column 5 indicates the sex (1=male, 2=female, 0=unknown), and column sex determines the affection status (2=affected, 1=unaffected, 0=unknown).

D1S303_AP2.abi - Editor

Datei Bearbeiten Format Ansicht ?

Marker	Lane	ID	A_1	A_2
D1S303	1	21	184	184
D1S303	2	22	184	184
D1S303	3	23	184	184
D1S303	4	24	184	184
D1S303	5	25	184	188
D1S303	6	26	184	190
D1S303	7	27	184	190
D1S303	8	28	190	190
D1S303	9	29	184	190
D1S303	10	34	184	190

D1S506_AP2.abi - Editor

Datei Bearbeiten Format Ansicht ?

Marker	Lane	ID	A_1	A_2
D1S506	1	21	136	136
D1S506	2	22	136	136
D1S506	3	23	136	136
D1S506	4	24	136	136
D1S506	5	25	134	136
D1S506	6	26	134	136
D1S506	7	27	134	136
D1S506	8	28	134	134
D1S506	9	29	134	136
D1S606	10	34	134	136

Fig.2.8: Marker files. Column 1 indicates the marker name, column 2 represents randomly chosen numbers, column 3 represents the person ID, and column 4 and 5 indicate the allele sizes that were achieved by the marker analysis.

2.4 Sanger sequencing

Exon primers for candidate genes were generated with Primer3 (for primer sequences see table 2.4, 2.5 and 2.6), ordered from Microsynth AG and diluted with LiChrosolv (Merck) to a final working concentration of 10µM. The *LMNA* Primers were stock primers, used for the diagnosis of CMT2B1 (Charcot-Marie-Tooth neuropathy) patients on the Institute of Human Genetics.

Table 2.4: *HR* Primer sequences for the amplification of exons 2 to 19.

Exon primer	Sequence	TM (in °C)
HR_E2_1f	5'GATGGTTATGCTCCAGGGAC	59,4
HR_E2_1r	5'CTATGCTCAGGCATCAGGG	59,4
HR_E2_2f	5'ATGGGGAGAGGAAGGTCAAC	60,3
HR_E2_2r	5'GCATCTTGGGGACCACC	59,8
HR_E3_1f	5'GTTCTGCCCATCCATTTAGG	59,4
HR_E3_1r	5'GCTGGCTCCACTGGCAC	61,7
HR_E3_2f	5'GCCACCAGCAACACCAAG	61,3
HR_E3_2r	5'CATAAAGCCTACAGACCCCG	59,6
HR_E4-5f	5'AGGTGAGGAATGGGGTTCTC	60,3
HR_E4-5r	5'CAGGGGAGGGACACCTG	60,0
HR_E6f	5'GTGAAGCCTTCCATTGGGG	62,7
HR_E6r	5'AATGACCACAGGCTTGCAG	59,8
HR_E7-8f	5'TCTCTGACCTTAACCTGTGATTACC	59,9
HR_E7-8r	5'CAAAGGTCAGCCATTTGCAG	61,7
HR_E9f	5'TCTGTTGAATTGTGTCTGCCAT	60,6
HR_E9r	5'GAGACTTCCGCGACTGTCC	61,0
HR_E10f	5'CCAGGAAGAGAGGGAAGAGC	60,5
HR_E10r	5'GGCACAGGGGCTTAGGAC	60,6
HR_E11f	5'ACGGAGGGAGGGCTAGG	60,2
HR_E11r	5'GAGCCACTGGGTCTGTCTG	59,4
HR_E12-13f	5'GTCCCCGAGCTGTTCTACTG	59,9
HR_E12-13r	5'AGGAGGGGAGGGCTGAAC	61,6
HR_E14f	5'TGATGGGGTCTCTGGTGC	60,7
HR_E14r	5'CGAGATGACAGGCAGACAGG	62,0
HR_E15-16f	5'TCTCCATGTTGAGGCTGGTC	61,2
HR_E15-16r	5'ACTGGACGAGCTTCTAGGGC	60,9
HR_E17f	5'CTCTGGAAAGTCCATGCCC	60,6
HR_E17r	5'GTCGCTTCTGCCATCCTG	60,5
HR_E18f	5'GGACAGGGAGAGGATGTGTG	60,5
HR_E18r	5'CACAGGGAGGTGGGAGG	60,0
HR_E19f	5'TTCCTTTTAAGTAGGGTGGGG	59,4
HR_E19r	5'CTGGGCTGAGCACCTGG	61,6
HR_E11f_V2	5'GAATACACATGGCCTTCGC	59,1
HR_E11r_V2	5'ACTGGGTCTGTCTGGGCCT	62,1
HR_E15-16f_V2	5'ATTACAGGCGTGAGCAACC	58,7
HR_E15-16r_V2	5'ACCTGTCTGTGCGAGTTGG	59,9

E=Exon, f=forward, r=reverse, V2= version 2, numbers indicate the exon of interest

Table 2.5: *P2RY5 (LPAR6)* primer sequences.

Exon primer	Sequence	TM (°C)
P2RY5_E1_1F	5'AATATTTATAAAACCATCCAAAGATCC	58,3
P2RY5_E1_1R	5'CACAGTTAACCACACGCCAG	60,2
P2RY5_E1_2F	5'TTGGCCATTTGGAGATTTAC	57,5
P2RY5_E1_2R	5'AGCAATTAACAAATGTTTGTGTTC	57,4
P2RY5_E1_3F	5'CTGGCGTGTGGTTAACTGTG	60,2
P2RY5_E1_3R	5'AGACATTACAGATTGGCACCAC	59,0

E=Exon, f=forward, r=reverse

Table 2.6: *LMNA* primer sequences (M13 universal primer sequences are added to the *LMNA* primers for faster sequencing set-up; M13 sequences are not included in the TM calculation)

Exon primer	Sequence	TM (C°)
LMNA_e1f	5‘CACGACGTTGTAAAACGACTCCGAGCAGTCTCTGTCCTT	60,1
LMNA_e1r	5‘GGATAACAATTTCACACAGGCCCTCTCACTCCCTTCCTG	63,6
LMNA_e2f	5‘GGATAACAATTTCACACAGGCTGGCACTGTCTAGGCACAC	58,4
LMNA_e2r	5‘CACGACGTTGTAAAACGACGGGAGGGCCTAGGTAGAAGA	59,7
LMNA_e3f	5‘CACGACGTTGTAAAACGACCCACCTCTCAGCTTCCTTCC	61,3
LMNA_e3r	5‘GGATAACAATTTCACACAGGAAGGCGAGCTCTGCACAC	60,3
LMNA_e4f	5‘CACGACGTTGTAAAACGACTAAAGTGGGGCTGGTAGTGG	60,0
LMNA_e4r	5‘GGATAACAATTTCACACAGGCTGATCCCCAGAAGGCATAG	59,6
LMNA_e5f	5‘GGATAACAATTTCACACAGGTAGCAGTGATGCCCAACTCA	60,4
LMNA_e5r	5‘CACGACGTTGTAAAACGACGCCATCTGACTCCACATCCT	60,1
LMNA_e6f	5‘GGATAACAATTTCACACAGGGTCCCTCCTTCCCCATACTT	59,3
LMNA_e6r	5‘CACGACGTTGTAAAACGACCCAAGTGGGGGTCTAGTCAA	60,0
LMNA_e7f	5‘GGATAACAATTTCACACAGGAGGTGCTGGCAGTGTCCTCT	62,4
LMNA_e7r	5‘CACGACGTTGTAAAACGACCTCTGAGGGCAAGGATGTTC	59,8
LMNA_e8+9f	5‘GGATAACAATTTCACACAGGTGGGCCTTTGAGCAAGATAC	60,2
LMNA_e8+9r	5‘CACGACGTTGTAAAACGACTCTAGAAAGGGGCCCTGAAT	60,0
LMNA_ e10+isof	5‘GGATAACAATTTCACACAGGTCACTGGGGTAGACATGCTG	59,7
LMNA_e10+isor	5‘CACGACGTTGTAAAACGACCAGGCCAGCGAGTAAAGTTC	60,0
LMNA_e11f	5‘GGATAACAATTTCACACAGGTGGGCCTGAGTGGTCAGT	60,3
LMNA_e11r	5‘CACGACGTTGTAAAACGACCGTCCTACCCCTCGATGAC	60,5
LMNA_e12f	5‘GGATAACAATTTCACACAGGGGGAGATGCTACCTCCCTTC	60,0
LMNA_e12r	5‘CACGACGTTGTAAAACGACGGGTTATTTTTCTTTGGCTTCA	59,5

E=Exon, f=forward, r=reverse, iso= isoform

The PCR was set up with:

6,0µl HotStarTaq Master Mix (5U/µl) (Qiagen)
4,0 µl RNase free H_20 (Qiagen)
0,5µl per 10µM Primer (M13 primers for LMNA sequencing)
1,0µl DNA (~50ng/µl).

Amplification was conducted under following cycling conditions:

Table 2.7: Cycling conditions for *HR, P2RY5 and LMNA* exon amplification. Note: The annealing temperature for P2RY5_E1_2 primers was 55°C.

1	94°C	15 min
2	95°C	25 sec
3	57°C (55°C)	30sec
4	72°C	1min
Go to step 2 →**34x**		
5	6°C	∞

After checking the PCR Products on a 1% LE-Agarose (Biozyme) (1g solved in 100ml of aqua bidest; staining with GelRed™ from Biotium), they were amplified in a 10µl reaction containing:

7,3µl LiChrosolv (Merck)
0,5µl BigDye Terminator 3.1 (Applied Biosystems)
1,4µl Sequencing Buffer (5x) (Applied Biosystems)
0,3µl 10µM forward or reverse exon primer or M13 primer (see table 2.8)
0,5µl PCR Product (~25-50ng)

M13 primer had following sequence:

Table 2.8: M13 Primer sequences

Primer	**Sequence**
M13f	5‘CACGACGTTGTAAAACGAC
M13r	5‘GGATAACAATTTCACACAGG

The sequencing reaction was conducted under following cycling conditions:

Table 2.9: Sequencing cycling conditions

1	95°C	30 sec
2	50°C	15 sec
3	60°C	4 min
Go to step1 →**24x**		
4	6	∞

Sephadex G-50™ Superfine (GE-Healthcare) and Centri-SEP columns (Applied Biosystems) were used for the removal of remaining dye remnants. For this purpose the whole sample was first diluted 1:1 with water. Sephadex columns were generated through centrifugation of the Sephadex filled Centri-SEP columns at 750rcf for 2 minutes to remove the interstitial water. Next the columns were put onto new elution strips, the entire diluted sequencing reaction was applied onto the fresh Sephadex columns and spun at 750rcf for 2 minutes. Samples were seperated on the ABI 3130*xl* Genetic Analyzer (with POP-7™ Polymer from Applied Biosystems).

The acquired sequences were visualized with ChromasLite software (available from http://www.technelysium.com.au/chromas_lite.html) and analyzed on UCSC genome browser (GRCh37/hg19) (Kent et al., 2002; Kent, 2002) by comparison with the reference sequence.

2.5 Validation of the mutation

After confirming mutations in a certain gene by sequencing, the possibility for being a SNP first had to be ruled out. This was done by aligning the acquired sequence to the USCS database (Kent, 2002) reference sequence and analyzing the dbSNP entries under the mutation locus. Next, OMIM (McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University (Baltimore, MD), 2012), HGMD (Stenson et al., 2003), and literature were screened for reports on the detected mutation. The segregation in the family was investigated by further sequencing of both, non-affected and affected family members.

3 Results

3.1 Family AP1

Patients in this family show the typical phenotype for the ‘woolly hair’ syndrome. Although the hair density appears normal, the growth is restricted to a very short period of time. In contrast to the tight, curly hair characteristic of Africans, this hair texture is rather fragile, extremely thin and kinky and the hair pigmentation is often reported to be below average (Shimomura, 2012; Shimomura et al., 2010; Shimomura et al., 2009).

3.1.1 Homozygosity mapping

Initially, individuals AP1-4 and AP1-12 were sent for the 250K SNP array since they result from two different branches of this family pedigree. Both parents of patient AP1-4 (left branch), AP1-1 and AP1-3, were the cousins of individual AP1-12 (right branch). However, the only homozygous region that could be detected was a 7Mb stretch on chromosome 11, but it did not share the same haplotype (see Fig. 3.1).

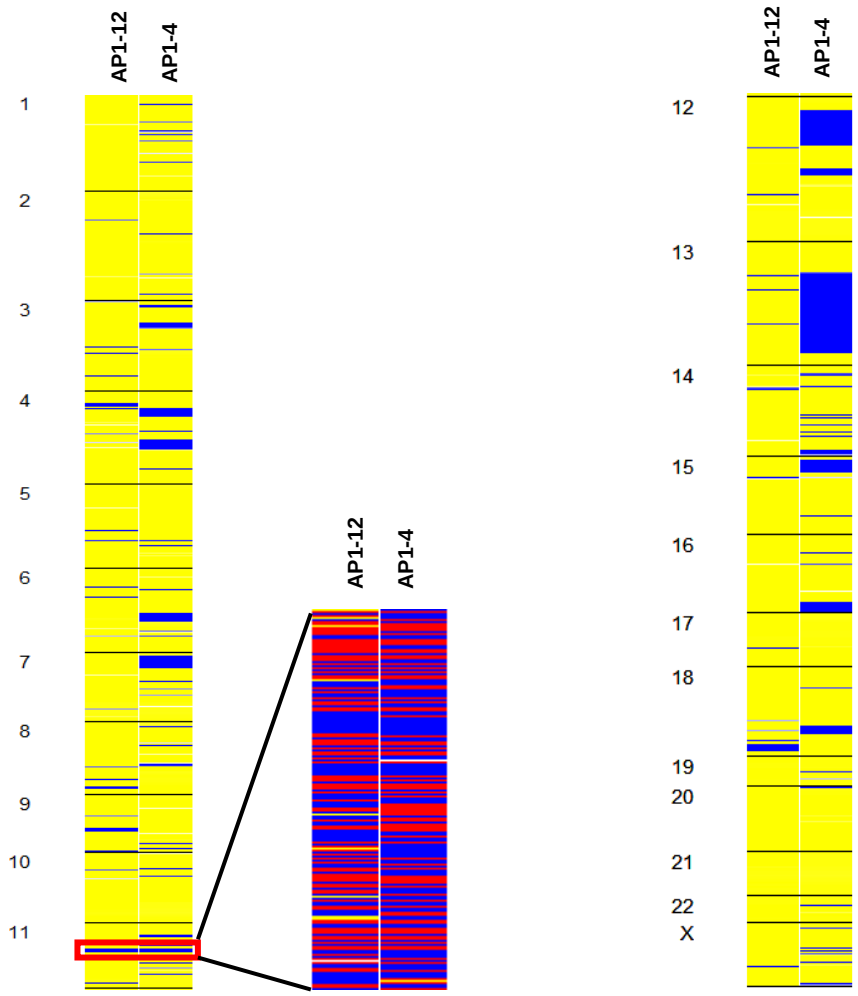

Fig. 3.1: 250K SNP array with patients AP1-12 and AP1-4. Chromosomes 1-22 plus X chromosome of individuals AP1-12 and AP1-4 reveal a single overlapping homozygous stretch on chromosome 11. Yellow indicates the heterozygous, blue the homozygous regions. The homozygous region on chromosome 11 does not display the same haplotype in the patients. Red=homozygous for A, blue=homozygous for B, yellow= heterozygous for A and B.

Therefore, the possibility of a compound heterozygosity arose, but a closer look at the haplotypes of the two individuals did not reveal any identical regions. However, since individual AP1-4 had more homozygous stretches than AP1-12, her affected brother, AP1-2, was further analyzed to check if at least the two siblings shared a homozygous candidate disease loci. Common homozygous stretches were detected between patients AP1-2 and AP1-4 on chromosome 4p15.1-q12 (31,7Mb-50Mb), 7p21.3 (8,4Mb-12,7Mb) and 13q14.11-q21.2 (42,1Mb-61,3Mb).

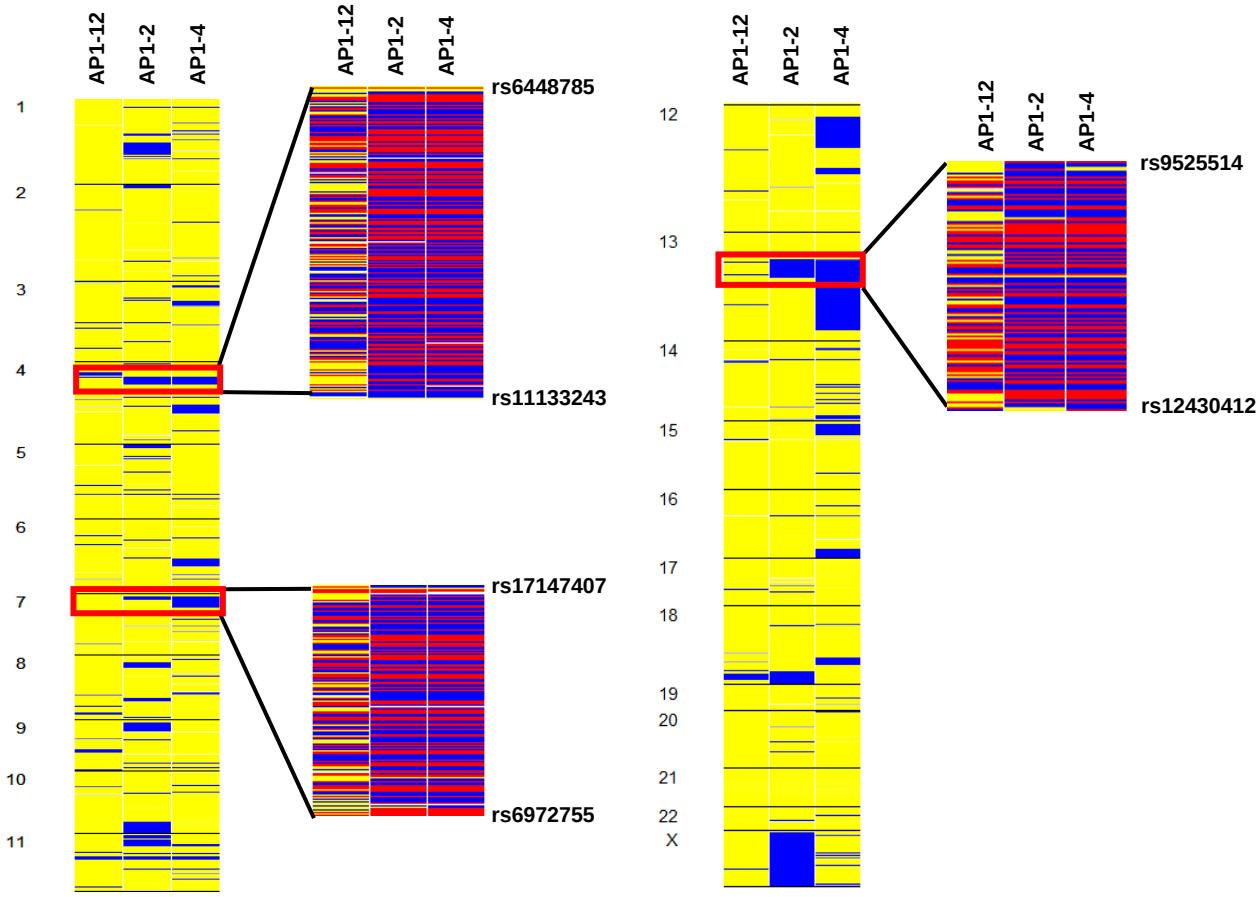

Fig.3.2: 250K SNP array from AP1-12, AP1-2 and AP1-4. The common homozygous regions from AP1-2 and AP1-4 are outlined with red squares and the haplotypes of these regions were magnified. The bordering SNP rs numbers of the shared homozygous stretches are written next to the haplotype view.

For the non-syndromic phenotype of autosomal recessive 'woolly hair' (ARWH), two genes have been reported so far: *LPAR6* (also known as *P2RY5*) (48,98 Mb) (OMIM 278150) on chromosome 13q14.2 and *LIPH* on chromosome 3q27.3 (OMIM 604379) (Kazantseva et al., 2006; Pasternack et al., 2008). In comparison to the SNP array results, the homozygous locus on chromosome 13 includes the *LPAR6* gene. The microsatellite marker analysis with markers D13S263 (42,98Mb) and D13S153 (48,79Mb) confirmed the segregation of this locus among the left branch family members. Although we could not detect homozygous haplotypes in the right branch of the family, all affected showed identical haplotypes for the same locus which would be expected for autosomal recessive traits in families without consanguineous background. We therefore speculated that a second mutation in a gene of the locus

on chromosome 13 might be present in one of the parents of the right branch (AP1-10 and AP1-9) independent from the consanguinity in the family.

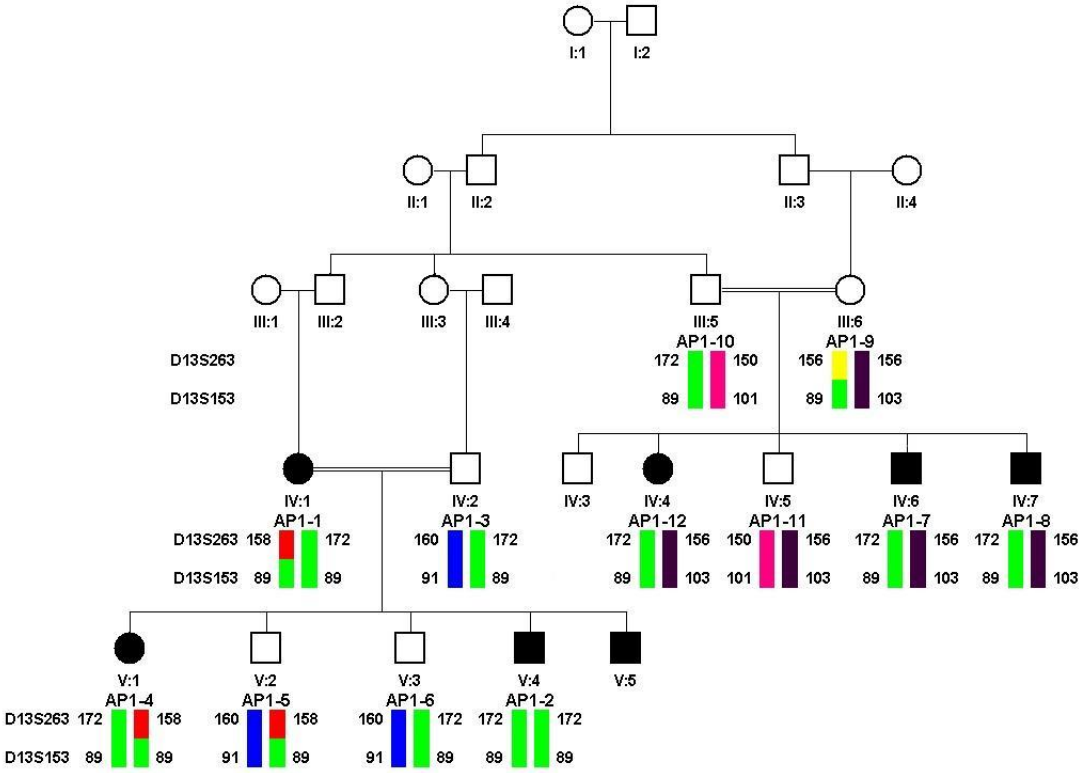

Fig. 3.3: Pedigree of family AP1 with the results from the segregation analysis of the woolly hair locus on chromosome 13q14.2 with the microsatellite markers D13S263 and D13S153. Marker D13S153 (48,79) is found to be homozygous among the affected in the left branch (allele size 89/89 indicated in green), whereas the segregation of the markers in the right branch indicates a compound heterozygosity in the affected (allele sizes 172/156 for D13S263 and 89/103 for D13S153). Double lines indicate consanguineous marriages.

It can be seen that the distal marker, D13S153, is present in both alleles of all affected individuals of the left branch with the same allele size (89bp), while the right branch patients, excluding the healthy individuals, always have allele 172/156 for D13S263 and 89/103 for D13S153. This latter observation suggested that the right branch patients might be carriers of a compound heterozygous or incidentally homozygous mutation in this gene, since they obviously also carry the suspected disease allele of the left branch (indicated in green in fig. 3.3).

3.1.2 Sanger Sequencing of *LPAR6/P2RY5*

Mutations in the *LPAR6/P2RY5* gene were confirmed by Sanger sequencing, revealing a homozygous c.565G>A (p.Glu189Lys) missense mutation in the left branch of the family. This same mutation, this time in a heterozygous state, was detected in all affected of the right branch along with another heterozygous c.188A>T (p.Asp63Val) transition. The compound heterozygosity was confirmed after it was shown that the first mutation was inherited from the father while the latter one descended from the mother.

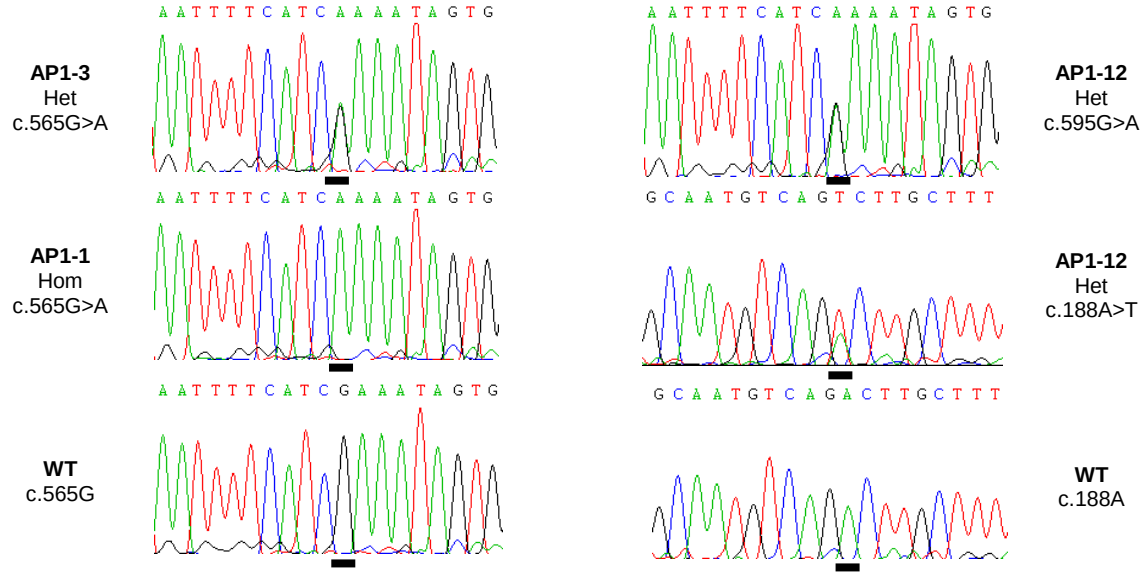

Fig. 3.4: Sanger sequences of *P2RY5* in family AP1. While affected individuals of the left branch were homozygous for c.565G>A, patients on the right branch carried two heterozygous mutations in the same gene, c.565G>A and c.188A>T. Positions of the mutated bases are indicated by black bars.

Both mutations can be found on HGMD (Stenson et al., 2003) and were first reported by Shimomura et al in 2008 (Shimomura et al., 2008).

3.2 Family AP2

In this family the scalp hair is short and kinky and thins out over time, resembling the hair disorder ‘hypotrichosis’ to a certain degree. According to the phenotype observed on the girl’s scalp, a stronger bolding pattern on the crown of the head can clearly be detected. Due to the shaved scalp hair, no statement can be made about the men concerning this pattern. Eyebrows and eyelashes are present but no information is available about the remaining body hair. The hyperkeratosis on the palm of the hands and feet are a feature of the siblings in the right branch, while neither pictures of the palms nor information thereof was received from patient AP2-1. However, it was not possible to link these symptoms to any disease already reported.

3.2.1 Homozygosity mapping

In this family all four patients were sent for the 250K SNP array. A single homozygous region that shared the same haplotype in all four affected could be detected on chromosome 1q21.3-q23.1 that is restricted by SNPs rs2335353 (154,63Mb) and rs4657146 (158,10Mb). Neither in OMIM (McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University (Baltimore, MD), 2012) nor in the HGMD database (Stenson et al., 2003) an entry for this locus related with hair disorders or hyperkeratosis could be found. The literature research also did not reveal any reports of hair loss linked to this locus.

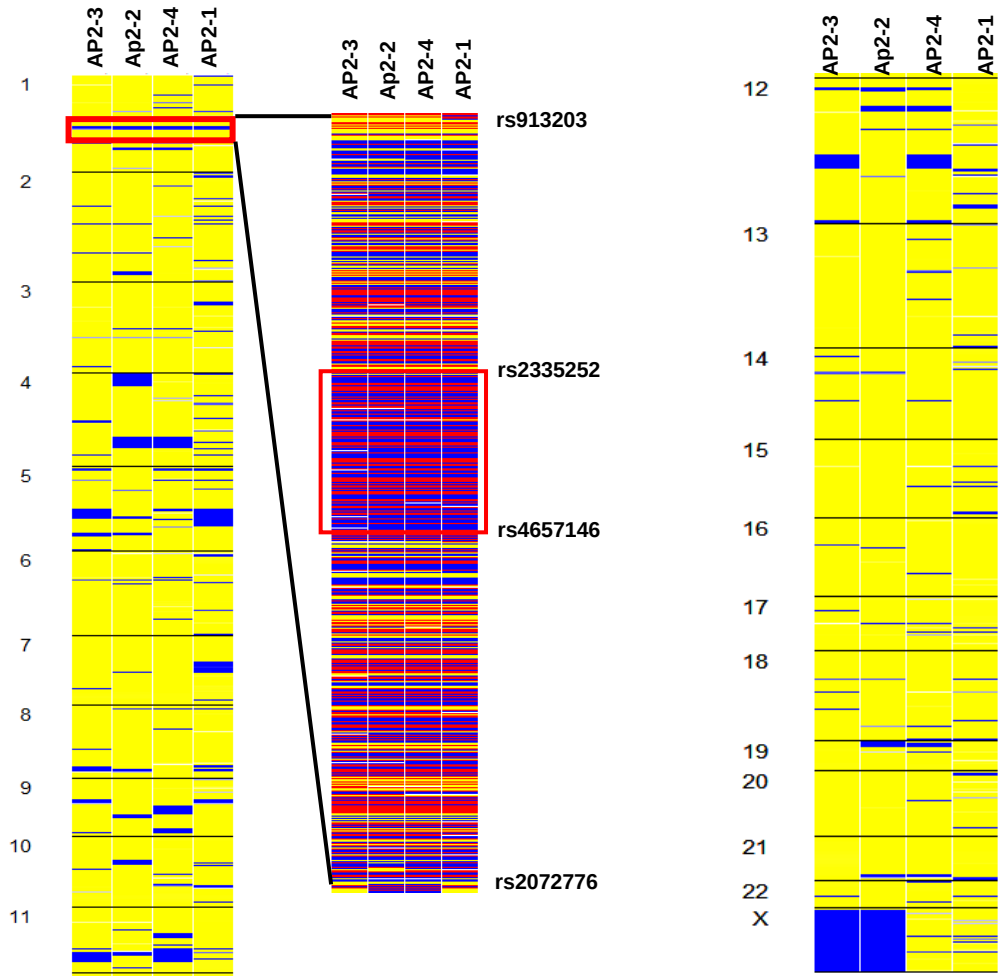

Fig. 3.5: SNP 250K array of individuals AP2-1, AP2-2, AP2-3 and AP2-4. The homozygous stretch on chromosome 1q21.3-q23.1 is outlined with a red square and shown at a magnified scale. It can be seen that the regions above and below the homozygous stretch (between rs913203 and rs2335252 as well as between rs4657146 and rs2072776) display the same heterozygous haplotype.

The microsatellite marker analysis with markers D1S303 (155,64Mb) and D1S506 (156,84Mb) confirmed the segregation of this locus among the family members since the affected were homozygous for the allele size 184 for marker D1S303 and 136 for D1S506. For the father II:5, we were able to estimate the haplotype of the right branch patients since his children were either homozygous for the green (patients) or the red allele (AP2-8) or heterozygous (AP2-7) for both.

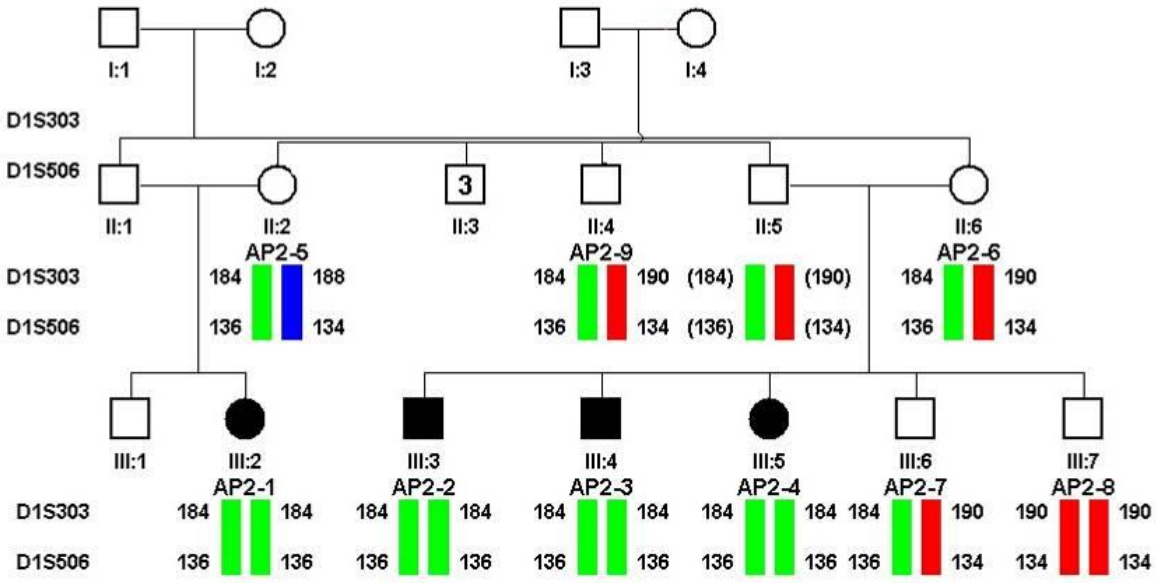

Fig. 3.6: Microsatellite marker analysis of chromosomal region 1q21.3-q23.1 in family AP2. The haplotype in green is clearly segregating with the desease status and occurs twice in the patients (filled black squares/circles), indicating an identical-by-descent region. The haplotype of II:5 was inferred (allele sizes indicated in parentheses) since we had no DNA from this individual.

In this family the pedigree includes only 3 generations, giving no clear information about the consanguinity of the marriages. Consanguinity can just be assumed due to the long homozygous stretches in the dChip analysis of the SNP array that would otherwise be unlikely to be found. To further investigate the possibility of a compound heterozygous mutation in a known hypotrichosis gene, we screened the array data for loci with the same haplotype shared by all affected. Five loci could be found, including two on chromosome 1 (see fig. 3.5) between 1p13.2 and 1q21.3 (116,01Mb-154,64Mb) as well as between 1q23.1 and 1q24.2 (158,10Mb-170,72Mb), on chromosome 8p23.3-8p23.2 (190,568bp-3,45Mb), 8q22.3-8q23.3 (102,30Mb-117,17Mb) and one more on chromosome 11p15.5-11p15.1 (211,447bp-17,79Mb). Again, none of these loci was reported for carrying an alopecia or hypotrichosis gene.

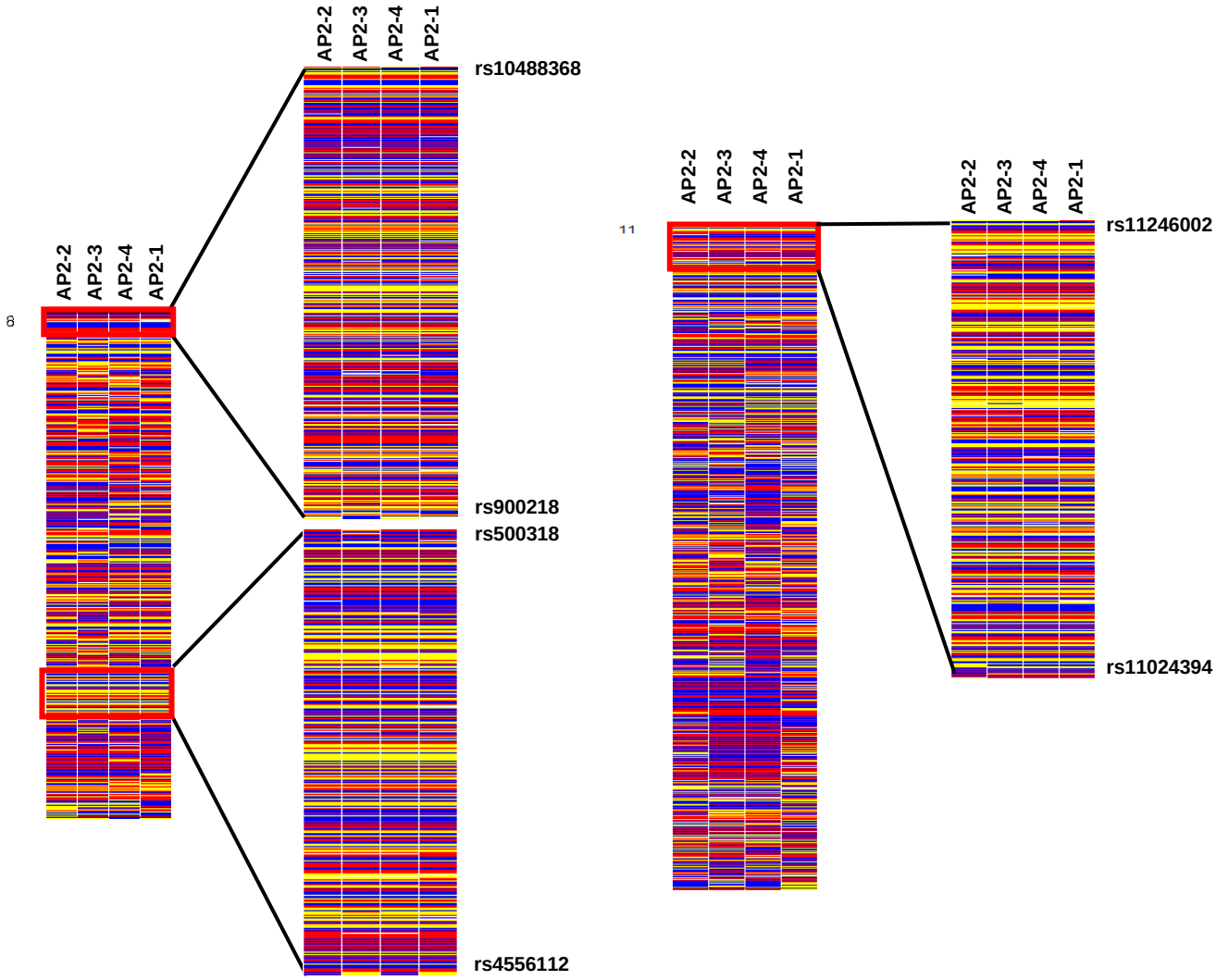

Fig.3.7: Possible compound heterozygous regions on chromosome 8 and 11 of family AP2. Regions of interest are outlined in red and magnified with the restricting SNPs indicating the end of the shared heterozygous regions.

Furthermore, AP2-3, if even at all affected, seems to have a much weaker phenotype compared to the other patients individually. Only a few millimetres of the re-growing, shaved scalp hair are visible on the pictures, but they suggest a rather normal hair density. If AP2-3 would be excluded from the analysis, 7 other loci, including one small homozygous and six possible compound heterozygous clusters, could be filtered (see table 3.1; pictures not shown). Also, these regions did not link to a known hair disease locus.

Table 3.1: Filtered SNP clusters that are shared between patients AP1-2, Ap2-2 and Ap2-4 but not AP2-3.

Chromosome	Haplotype	Restricting SNPs	Chromosomal position (bp)	Chromosomal region
3	Het	rs1502616-rs7649638	59,530,321-61,960,972	p14.2
5	Het	rs764221-rs6894609	167,749,955-180,696,889	q34-q35.3
8	Het	rs2406991-rs6989785	3,496,223-5,041,331	p23.2
8	Het	rs6988624-rs1424883	64,931,948-72,597,686	q12.3-q13.3
8	Het	rs1463254-rs1385229	82,807,249-96,771,547	q21.13-q22.1
9	Het	rs9299046-rs11788817	7,390,841-15,011,868	p24.1-p22.3
21	Hom	rs2183589-rs2839373	46,821,491-48,129,895	q22.3

Het=heterozygous; Hom=homozygous

Since microsatellite marker analysis of all loci would have been rather expensive, we sent two non-affected family members, the brothers AP2-7 and Ap2-8, for the 250K SNP array analysis. This analysis decreased the number of filtered loci to four for AP2-1, Ap2-2, Ap2-3 and Ap2-4 (see table 3.2) and also to four loci that shared the same haplotype in the case that individual Ap2-3 is excluded from the analysis (see table 3.3).

Table 3.2: Filtered candidate disease loci for patients AP1-2, Ap2-2, Ap2-3, and Ap2-4 after comparing their haplotypes with those of the healthy relatives, Ap2-7 and Ap2-8.

Chromosome	Haplotype	Restricting SNPs	Chromosomal position (bp)	Chromosomal region
1	Hom	rs1131820-rs4657146	154,744,852-158,095,159	q21.3-q23.1
1	Het	rs4657146-rs2072776	158,095,159-168,169,452	q23.1-q24.2
8	Het	rs10488368-900218	190,568-3,454,659	p23.3-p23.2
11	Het	rs11246002-rs11024394	211,447-17,794,438	p15.5-p15.1

Het=heterozygous; Hom=homozygous

Table 3.3: Possible disease loci for individuals AP1-2, Ap2-2, and Ap2-4 after comparing the haplotypes of the affected with those of the healthy individuals, Ap2-7 and Ap2-8.

Chromosome	Haplotype	Restricting SNPs	Chromosomal position (bp)	Chromosomal region
5	Het	rs884599-rs6894609	171,129,907-180,696,889	q35.1-q35.3
8	Het	rs2406991-rs6989785	3,496,223-5,041,331	p23.2
8	Het	rs1463254-rs7014625	82,807,249-93,293,461	q21.13-q21.3
9	Het	rs9299046-rs11788817	7,390,841-15,011,868	p24.1-p22.3

Het=heterozygous; Hom=homozygous

3.2.2 Copy number variation plot

Although not very likely according to the inheritance pattern, the possibility for a copy number variation and loss-of-heterozygosity of the affected was investigated via dChip analysis. However, as expected, the copy numbers did not vary.

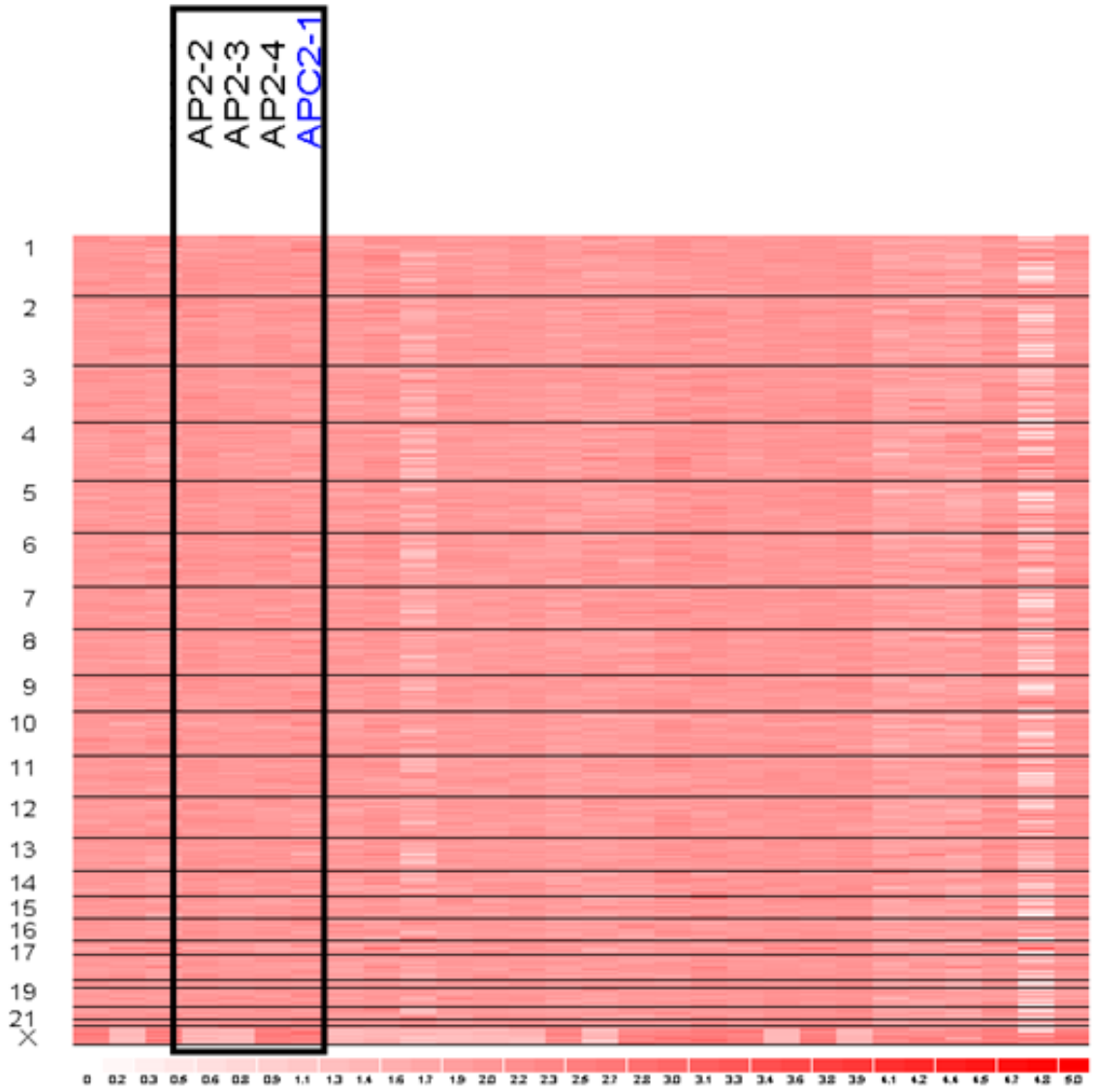

Fig. 3.8: LOH and copy number calculation of patients Ap2-1, 2-2, 2-3 and 2-4 with dChip. The varying red bar on the bottom indicates the possible copy numbers changes that could be detected with this analysis. 24 anonymous arrays from non-alopecia patients were used for normalization of the data. 2,0 represents a normal diploid amount. In male individuals, for instance, just one X chromosome is present resulting in a lighter (1.1) red. The affected AP2 patients are outlined with a black square; the other individuals were required for the normalization of the data.

3.2.3 LOD score calculation

For the homozygous locus on chromosome q21.3-q23.1 the LOD score was calculated with the marker information obtained from the microsatellite marker analysis. Since only two markers were used, only a limited number of analysis methods were possible. Multipoint LOD score analysis with GeneHunter generated a LOD score of 2,10 while the SuperLink v1.6 calculation (two-point analysis) was slightly lower for marker D1S506, which had an LOD score of 2,01 at a recombination fraction of ϴ=0.

3.2.4 Candidate gene approach

Genes within the homozygous 4Mb area on chromosome 1 were investigated to determine if they were involved in the hair cycle or hair follicle morphogenesis. However, although more than 80 genes reside within the filtered area (see fig. 3.9), none of them was clearly connected to the hair so far.

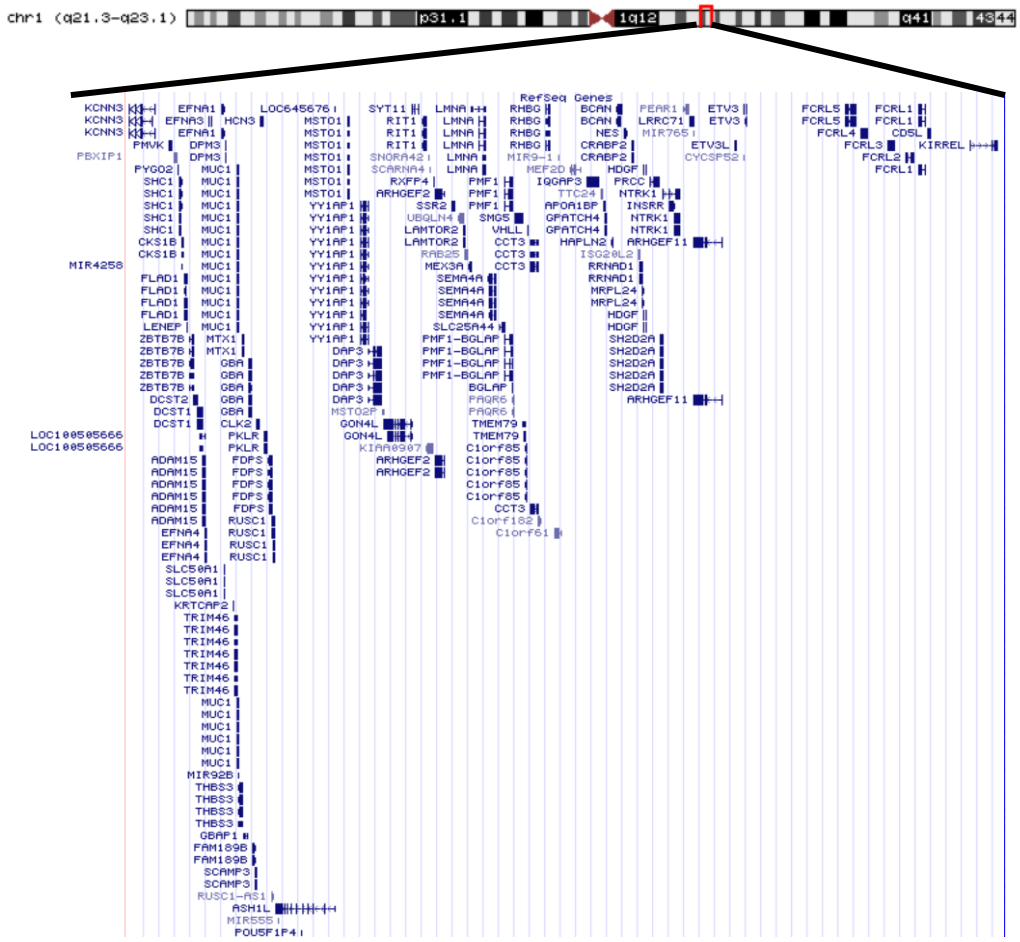

Fig. 3.9: UCSC RefSeq genes that are located within the homozygous stretch on chromosome 1.

Only *LMNA*, a gene that is known to cause Charcot-Marie-Tooth 2 (De Sandre-Giovannoli et al., 2002), could be regarded as a strong candidate gene, although the diseases resulting from mutations in this gene are much more severe than hair loss. For one disease caused by *LMNA* (chr.1: 156,08Mb-156,11Mb) mutations, namely lethal restrictive dermopathy, sparse to absent eyebrows, eyelashes and lanugo hair were reported in OMIM (OMIM 275210). Although the overall phenotype of this disease was not comparable to our patients' symptoms, the high amount of different diseases resulting from base pair variations in this gene plus the influence on some body hair in restrictive dermatopathy enforced the decision to sequence this gene. This assumption, however, turned out to be wrong after no such mutation, excluding four SNPs (rs538089, rs534807, rs505058 and rs7339) with rather high allele frequencies, was found in the *LMNA* gene.

3.3 Family AP3

The short, sparse, thin and slightly curly hair in combination with normal to sparse eyebrows in the affected patients of this family is a typical feature of autosomal recessive hypotrichosis, reported to be caused by mutations in the *LIPH* or *LPAR6* gene (Kazantseva et al., 2006; Pasternack et al., 2008).

3.3.1 Homozygosity mapping

Only patient AP3-3 was sent for the 250K SNP array. Two bigger homozygous stretches were filtered; a 12Mb region on chromosome 4q34.1-4q35.11 (172,96Mb-184,19Mb) and a prominent 36Mbp long homozygous stretch on chromosome 13q13.3-13q22.1 (38,21Mb-74,37Mb), including the LAH3 (localized autosomal recessive hypotrichosis) locus on 13q14.2 with the *LPAR6* (*P2RY5*) gene (Pasternack et al., 2008; Wali et al., 2007).

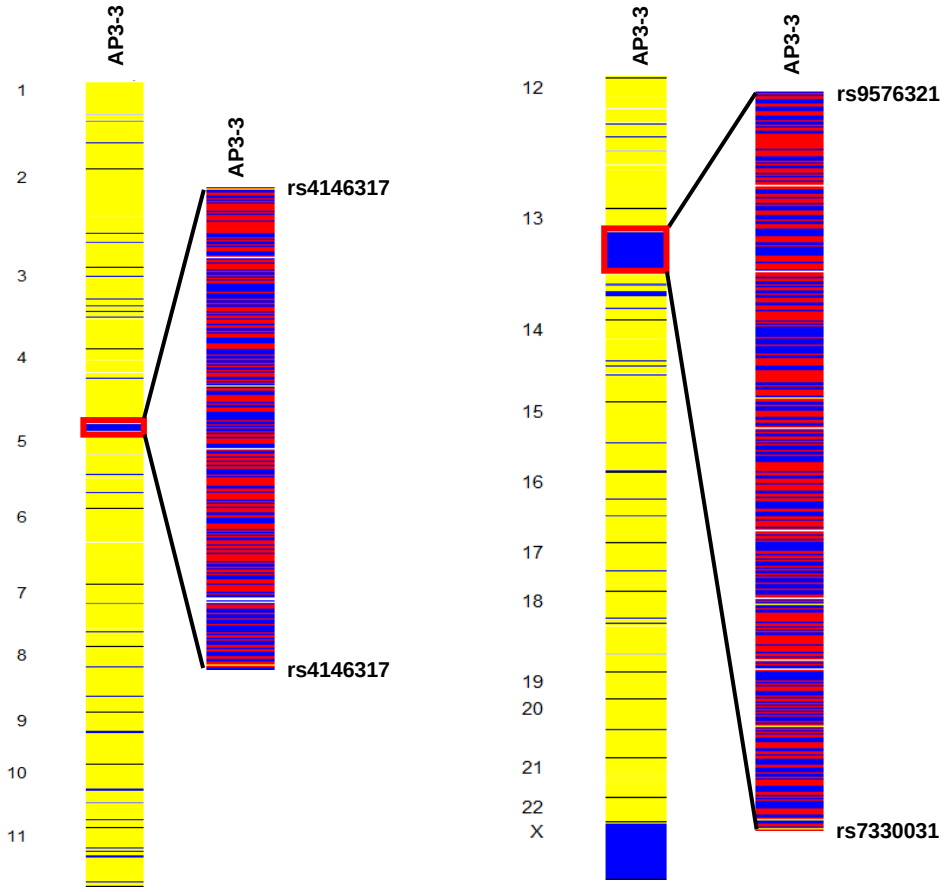

Fig. 3.10: 250K SNP array dChip analysis of individual AP3-3. Two loci on chromosome 4 and 13, were filtered. The homozygous stretch in the X chromosome indicates the male gender since men have only one X chromosome and are thus hemizygous for all genes located in this area.

The familiar distribution of the locus on Chromosome 13 was analyzed with polymorphic microsatellite markers D13S218 (39,03Mb), D13S263 (42,98Mb), D13S153 (48,79Mb) and D13S156 (74,66Mb) and confirmed the segregation of the region between marker D13S263 and D13S153 among the affected family members.

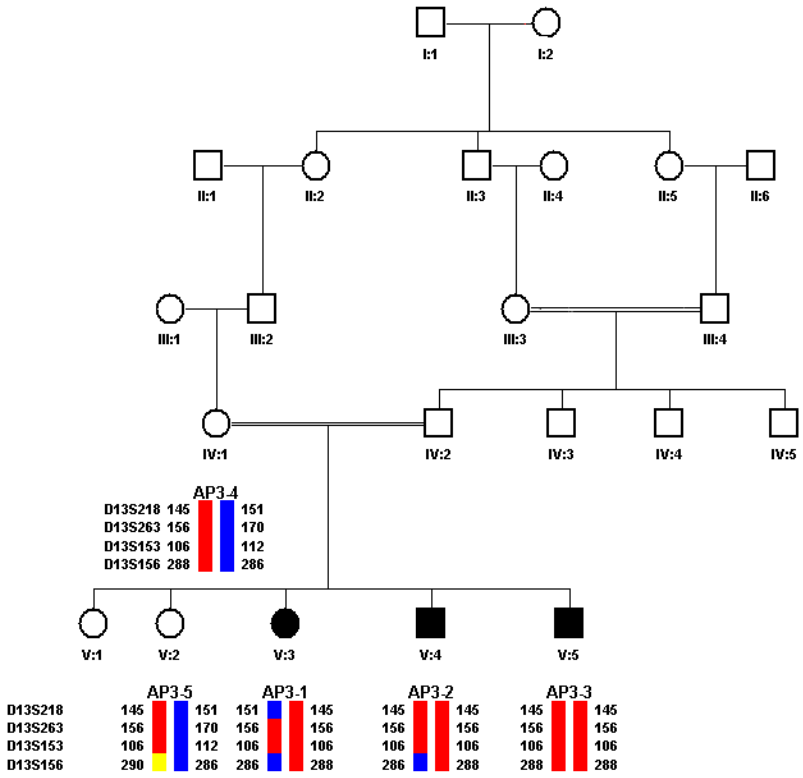

Fig. 3.11: Microsatellite marker analysis of the homozygous region on chromosome 13q13.3-13q22.1 in family AP3. It can be seen that the markers D13S263 and D13S153 are present twice with the same allele size (red bar) in the patients AP3-1, Ap3-2 and AP3-3, indicating an identical-by-descent region.

3.3.2 Sanger Sequencing of *LPAR6/P2RY5*

Further sequencing of the *P2RY5* gene confirmed a duplication of four bases, c.64_67dupTGCA, that lead to a frameshift resulting in a premature stop codon 28 amino acids (aa) after the first aa change (p.Phe24Hisf*s28). Literature research showed that the same mutation in an Indian family was already detected in 2009 by Pasternack et al (Pasternack et al., 2009).

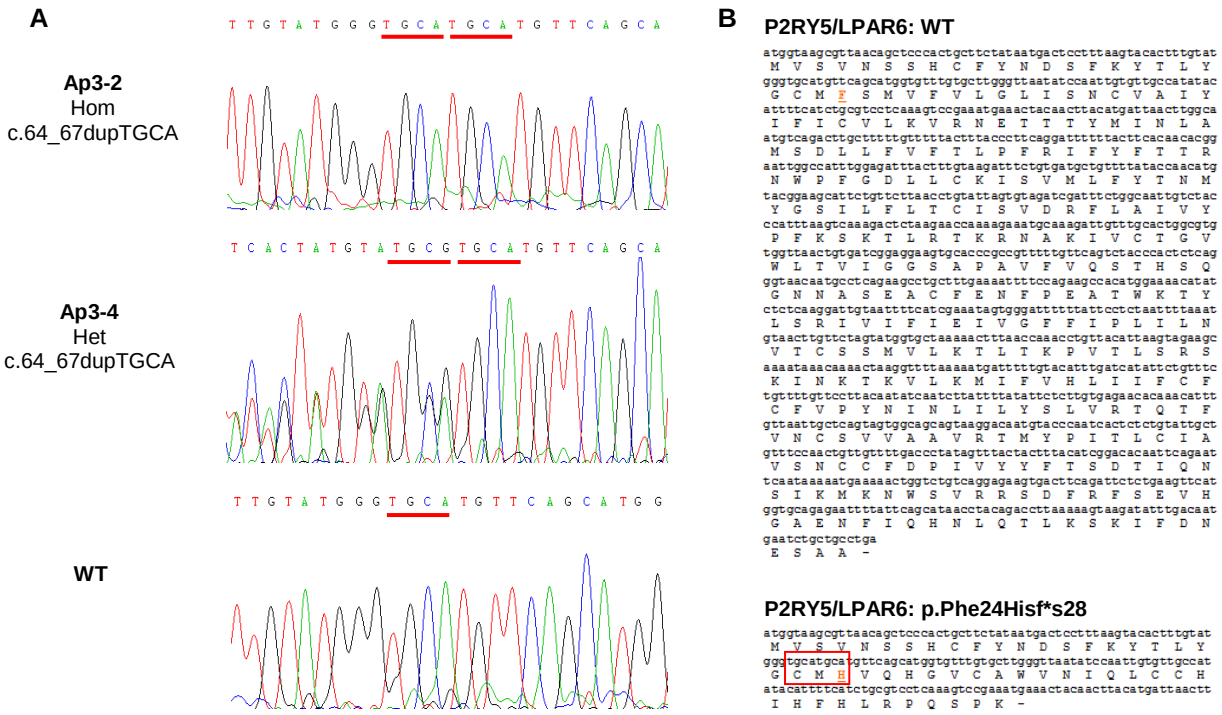

Fig.3.11: Sanger sequencing of *P2RY5* in family Ap3 revealed a duplication of TGCA between position 64 and 67. A. The resulting frameshift is detected as an overlapping sequence region in the heterozygous carrier, mother AP3-4. All sequences are shown in the 'reverse and complement' view since the forward primer did not give utilizable sequences. The duplicated bases are underlined in red. B. The duplication (outlined with a red square) also influences the aa composition, resulting in a premature stop codon 28 aa after the first mismatching aa. The first differing aa's are underlined and indicated in red. Aa translation was generated using the ExPASy translation tool (http://web.expasy.org/translate/) (Gasteiger et al., 2003).

3.4 Family AP4

Congenital loss of entire body hair after the postnatal ritual shedding is a very rare disease for which only two genes have been reported so far, namely *HR* (*hairless*) and *VDR* (*Vitamin D receptor*). Other than the loss of the entire body and scalp hair, no other symptoms have been reported for this family. For this family, it needed to be taken into account that mutations in the hairless gene only lead to the isolated bolding hair phenotype that sometimes appears along with keratin-filled cysts, while mutations in the *VDR* gene also cause hereditary vitamin D resistant rickets (Ahmad et al., 1998a; Miller et al., 2001; Sprecher et al., 1998). Patients in this family do not show any other abnormalities but congenital alopecia with countable eyebrows and eyelashes among the younger ones.

3.4.1 Homozygosity mapping

The affected son, Ap4-3, was sent for the 250K SNP array mapping. In the dChip analysis, sixteen larger homozygous stretches were detected (see table 3.4) including one 14Mb region on chromosome 8p22-8p12 (15,01Mb-29.36Mb).

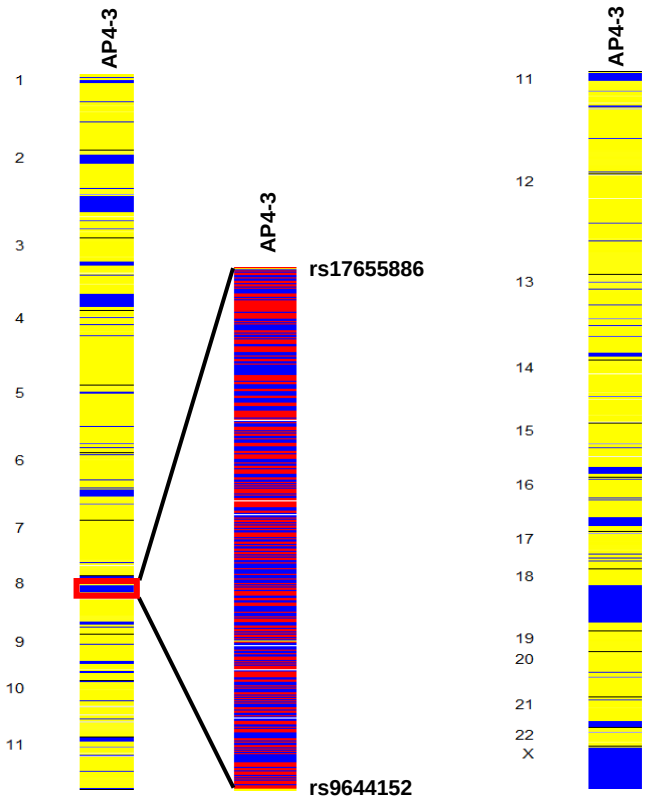

Fig.3.12: 250K SNP array analysis in dChip of patient AP4-3. The homozygous cluster on chromosome 8p22-8p12 is outlined with a red square and the haplotype with the restricting SNPs is magnified.

Table 3.4: Homozygous loci filtered in the 250K SNP array of patient AP3-4.

Chromosome	Restricting SNPs	Chromosomal position (bp)	Chromosomal region
2	rs181130-rs150658	8,273,020-33,180,619	p25.1-p22.3
2	rs10496652-rs10930354	126,736,747-170,273,127	q14.3-q31.1
3	rs17031633-rs6791324	60,727,326-67,437,836	p14.2-p14.1
3	rs9879590-rs13064299	150,325,498-187,865,100	q25.1-q27.3
6	rs4707664-rs2253310	91,963,759-108,888,593	q15-q21
8	rs10488368-rs2572406	190,568-11,092,252	p23.3-p23.1
8	rs17655886-rs9644152	15,012,123-29,362,563	p22-p12
8	rs17644857-rs7835363	115,566,620-121,380,382	q23.3-q24.12
9	rs11139400-rs4744106	84,373,209-92,873,665	q21.32-q22.2
9	rs10121864-rs10858376	133,548,406-138,158,757	q34.12-q34.3
11	rs757091-rs4910325	2,440,989-11,368,527	p15.5-p15.3
13	rs9300981-rs4291792	105,642,278-109,323,612	q33.2-q33.3
15	rs13380271-rs11856664	88,499,185-96,983,509	q25.3-q26.2
16	rs4506930-rs7196826	76,596,858-83,099,707	q23.1-q23.3
18	rs1467232-rs7238322	25,027,216-68,491,890	q12.1-q22.2
21	rs2212603-rs2839373	39,931,009-48,069,930	q22.2-q22.3

The homozygous cluster on chromosome 8 includes the *hairless* gene, known to be responsible for ‘universal congenital alopecia’ (or ‘congenital atrichia’) (Ahmad et al., 1998a; Sprecher et al., 1998).

No microsatellite marker analysis was performed for this family. The *HR* gene was sequenced directly.

3.4.2 Sanger Sequencing of the *HR* gene

The patients of this family were diagnosed to carry a nonsense mutation, c.2818C>T, in exon 13 of the *HR* gene, which at this position substitutes the arginine (CGA) for the stop codon TGA (p.Arg940X). This mutation has been reported in 2007 by Kim et al (Kim et al., 2007) for an ‘atrichia with papular lesions’ family.

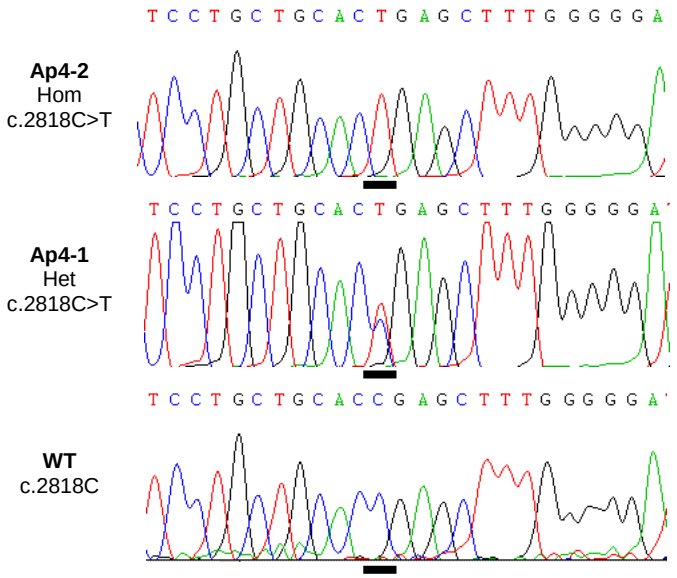

Fig. 3.13: Sanger Sequencing of the *HR* gene revealed the stop codon generating nonsense mutation c.2818C>T in exon 13. The mutated base is underlined with a black bar. The affected son, AP4-2, and the heterozygous father, AP4-1, are shown.

3.5 Family AP5

As for the patients in family AP4, the affected in this family display the same phenotype but in combination with little cysts on the skin. Again, no bone or other abnormalities were detected in the patients.

3.5.1 Homozygosity mapping

Affected second grade cousins, AP5-2 and AP5-6, were sent for the 250K SNP array. Two bigger, overlapping homozygous stretches that displayed the same haplotype, one on chromosome 8p23.1-8p21.3 (10,39Mb-23,25Mb) and another one on chromosome 19q13.12-19q13.2 (35,61Mb-41,13Mb), were found.

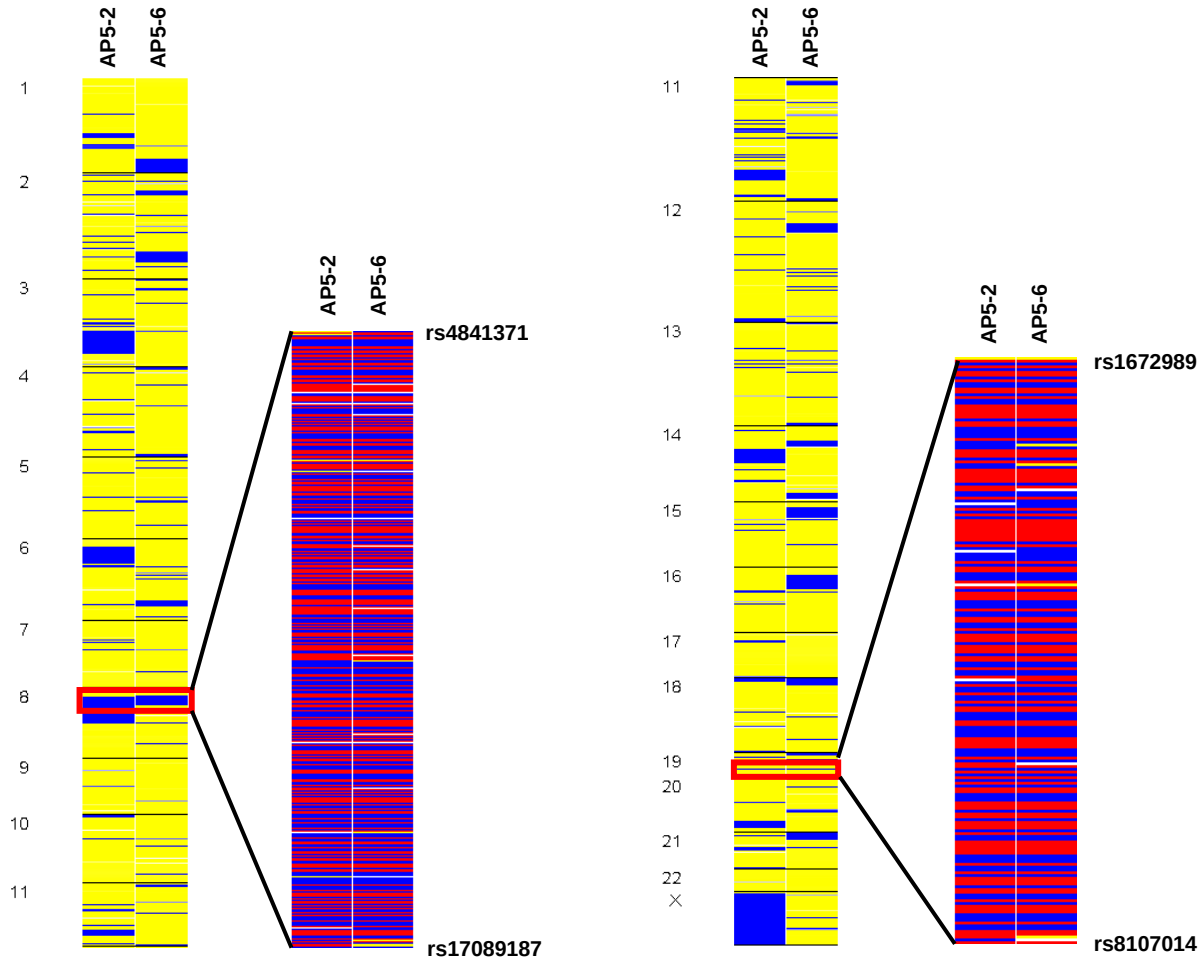

Fig. 3.14: dChip analysis of the 250K SNP array of patients AP5-2 and 5-6. The overlapping homozygous regions on chromosome 8 and 19 are magnified with the restricting SNPs displayed next to the ends of the homozygous clusters.

As expected, the cluster on chromosome 8 includes the congenital universal alopecia locus at 8p21.2. The microsatellite marker analysis confirmed the segregation of marker D8S258 (20,37Mb) among the affected, which are homozygous for the green allele.

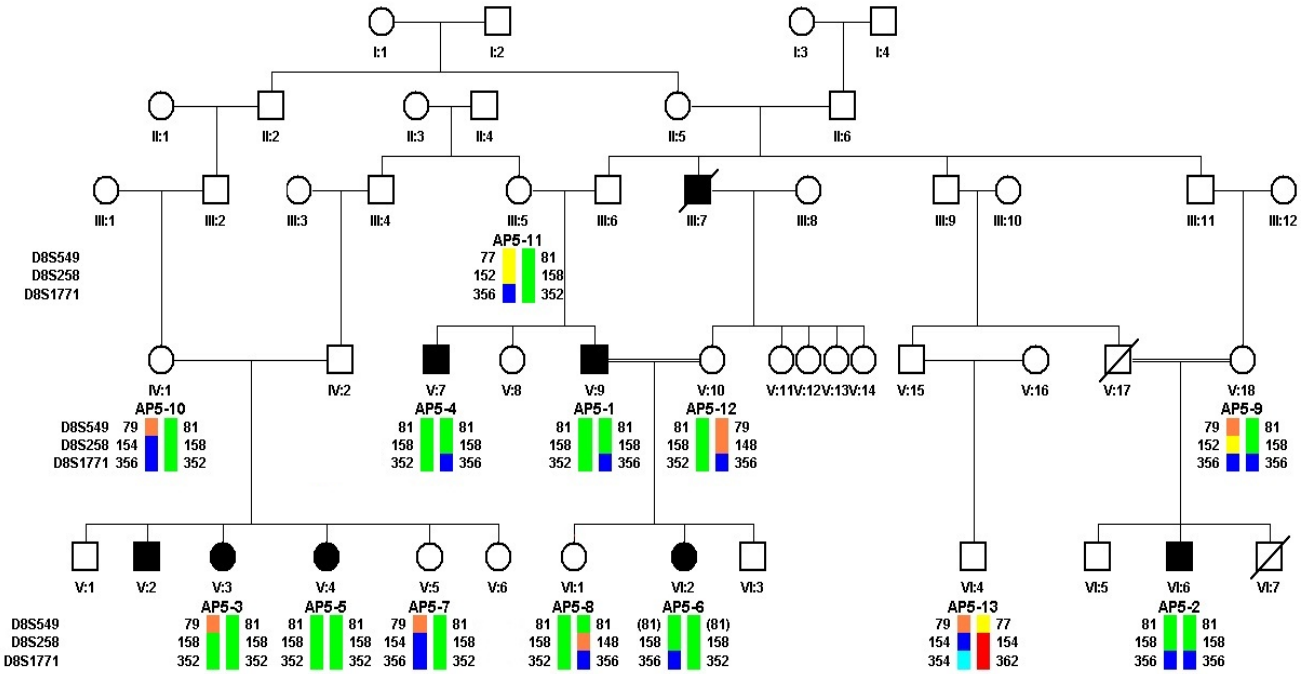

Fig. 3.15: Pedigree of family AP5 with the results from the microsatellite marker analysis. Only marker D8S258 segregates in a disease-specific manner, indicated by a green bar with the allele size 158/158 for the affected. Bars with different colours represent crossing-overs that have occurred between two markers. For individual AP5-6, the analysis for marker D8S549 was not successful and is thus indicated in parentheses representing an inferred marker size.

For the patients suffering from atrichia, the green allele for marker D8S258 can be found twice.

3.5.2 Sanger sequencing of the *HR* gene

Because the *hairless* gene resides within the segregating chromosomal area (21,97Mb), a sequencing approach was conducted and a deletion of the first adenosine marking the start of exon 8 was found. Although several mutations have already been reported in this gene (47 are documented alone on the HGMD website (Stenson et al., 2003)), the c.2123delA cannot yet be found in any literature.

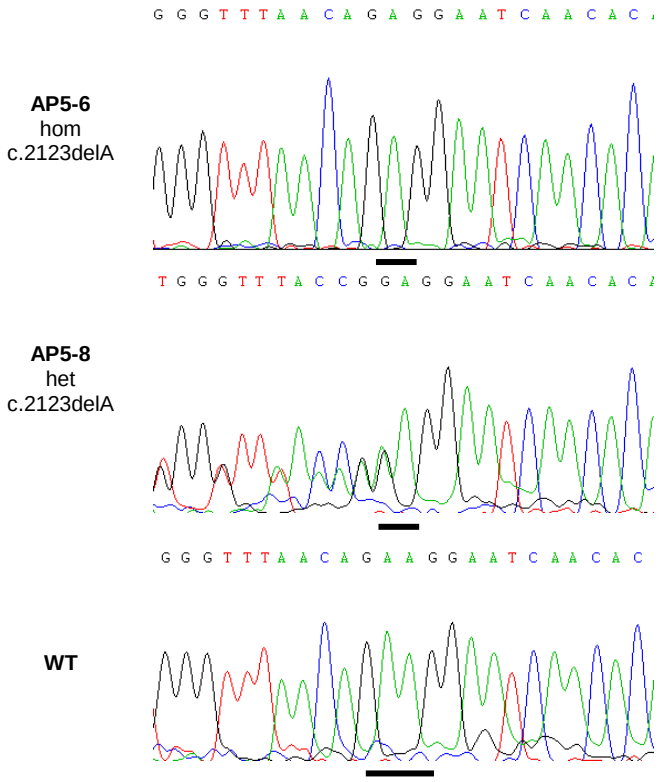

Fig. 4.16: DNA sequences of exon 8 displaying the deletion of the adenosine at c.2123 of the *HR* gene. The deleted base is underlined in black. All sequences are shown in the 'reverse and complement' view of ChromasLite since the forward sequence started too closely next to the deletion and did not show a good resolution.

Moreover, as a consequence of the changed frame, a premature stop codon can be found at 194 instead of 483 aa's after the first aa that differs from the original sequence (p.Lys708Argfs*194). (see appendix)

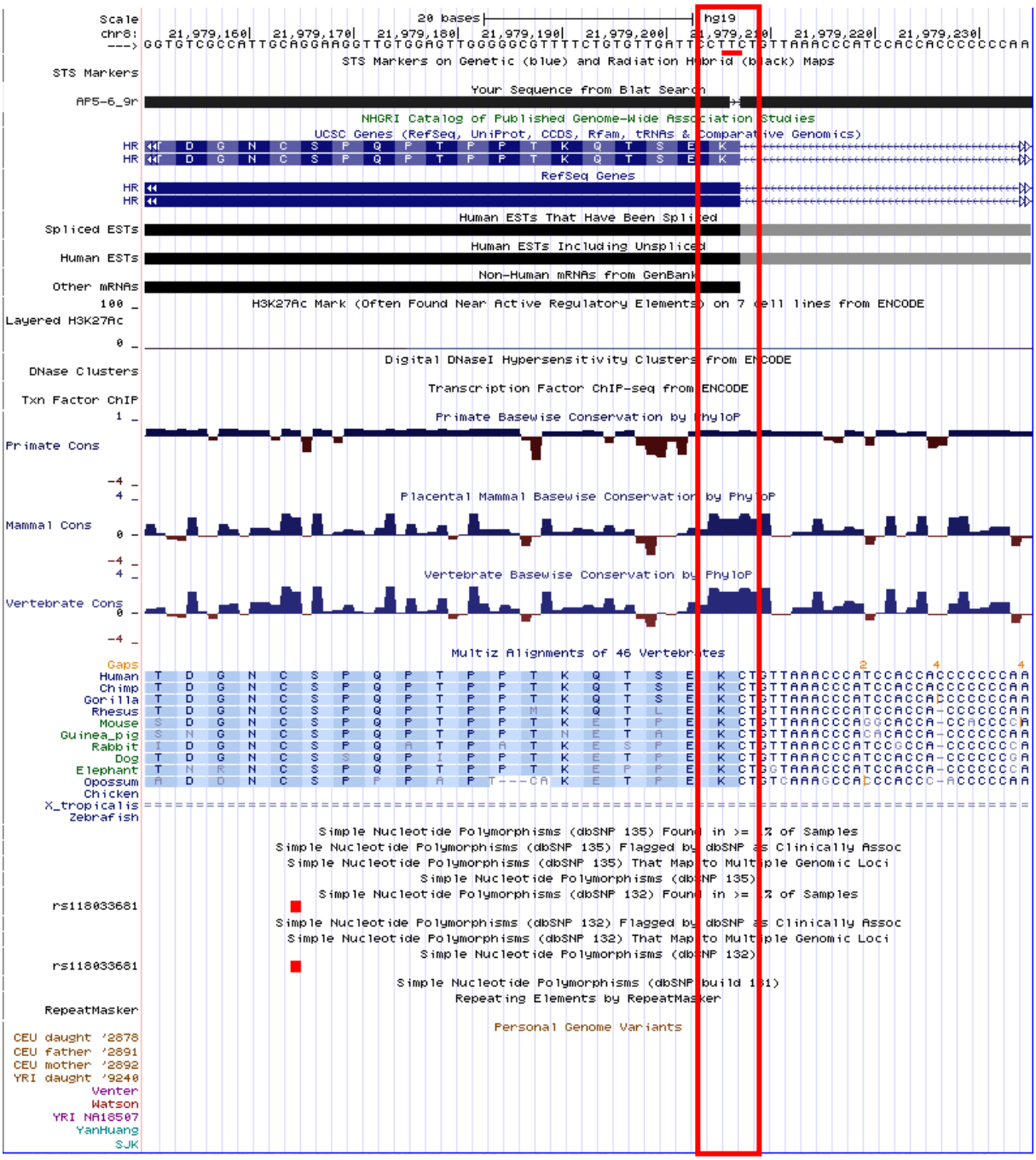

Fig. 4.17: Alignment of the *HR* exon 8 sequence of patient AP5-6 to the UCSC reference sequence. A deletion of the first or second 'A' ('T' on the chromosome, since the gene is transcribed in the reverse direction) is revealed (underlined in red). Moreover, on the very bottom of the browser window no SNP entry can be found (whole area indicated in a red square).

4 Discussion

The murine *hairless* gene was already identified and sequenced years ago in the *nude* mouse that initially possesses normal hair, but then loses it some weeks after birth. Moreover, their skin was also observed to be more sensitive. After exposure to UV light, for instance, they developed more frequent skin neoplasms compared to WT mice (Cachon-Gonzalez et al., 1994; Benavides et al., 2009). In 1999, Ahmad et al discovered mutations in the human homologue in patients suffering from universal loss of natal hair that did not grow back. They cloned the human *hairless* gene with the help of the murine cDNA sequence and mapped it to the short arm of human chromosome 8 (Ahmad et al., 1999).

Three different hair phenotypes resulting from *HR* mutations can be distinguished: if patients are only devoid of entire body hair and no ectodermal abnormalities are detected, alopecia universalis congenital, or 'congenital atrichia', is diagnosed. In the case of total alopecia in combination with little keratin-filled cysts on the skin that appear in the first decade of life, it was suggested to be called 'atrichia with papular lesions' (Ahmad et al., 1998b; Zlotogorski et al., 2001; Klein et al., 2002). Sometimes, but not always, white pigmented streaks are also reported (Zlotogorski et al., 2001; Klein et al., 2002). Another disease associated with mutations in the U2 region of the 5'UTR (*U2HR*) of the *HR* gene is the autosomal dominant 'Maria Unna hypotrichosis' or 'generalized hypotrichosis' (Wen et al., 2009; Suga et al., 2011). Affected of this disease are born with sparse hair and from then on develop only wiry and coarse hair that is shed during adolescence (Wen et al., 2009). All these different phenotypes indicate that the diseases resulting from *hairless* mutations are rather heterogeneous. The phenotype resulting from mutations in this gene is often mistakenly diagnosed as alopecia universalis, a form of the autoimmune disease alopecia areata, in which an infiltration of CD4+ and CD8+ T-cells, as well as natural killer cells, dendritic cells, and mast cells into the bulbar follicle epithelium occurs (Gilhar et al., 2012; Zlotogorski et al., 2001). This happens as a response to a high expression of normally downregulated follicular HLA class I and II antigens (Botchkarev, 2003). Interestingly, these inflammatory cells only attack the pigment-producing anagen hair follicle and put the hair follicle into a premature catagen phase. The onset of alopecia areata can happen at any age but is uncommon in the first three years of life (Gilhar et al., 2012). In contrast, mutations in the *hairless* gene inhibit the re-growth of hair

after the ritual shedding that happens up to 24 months after birth, an in this case no inflammatory infiltrates in the histological observation of the scalp skin can be found (Ahmad et al., 1998a; Sprecher et al., 1998; Zlotogorski et al., 2001).

There are two isoforms reported for the human *HR* gene, with its longest product consisting of 1189 aa while the shorter one lacks exon 17 (aa's 1072-1126). Although it was first stated that the shorter isoform was expressed in skin only (Cichon et al., 1998), Malloy et al (Malloy et al., 2009) proved the presence of both HR isoforms in skin and keratinocytes.

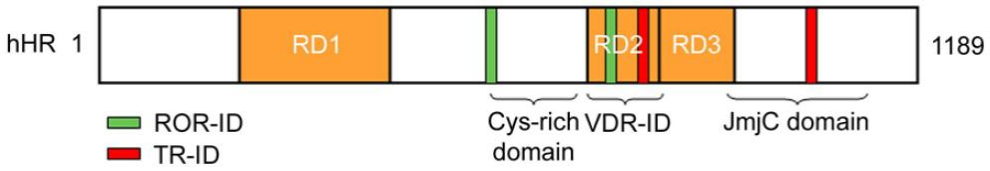

Fig.4.1: Organization of the human *hairless* gene. Its receptor co-repressor action is mediated through three domains, indicated in orange. The thyroid hormone receptor interaction domains are indicated in red, while the two RORα interaction domains are indicated in green. The Vitamin D receptor recognition domain (730–844 aa) lies within the repressor domain 2. In the RD1 a short nuclear localization signal (412-430 aa) is located (not shown) (Mi et al., 2011). Several cystein residues (567–692 aa) let Cahon-Gonzalez et al (Cachon-Gonzalez et al., 1994) assume that it is this gene encodes for a putative zinc-finger protein. The JmjC domain (946–1156 aa) on the C-terminal domain is often found in proteins with histone demethylase activity and mutations in the hairless gene in this domain prohibited the localization of the protein to MAD bodies (Mi et al., 2011). RD= repressor domain, TR-ID= Thyroid hormone receptor interaction domain, VDR-ID= Vitamin D receptor interaction domain. Figure adapted from (Thompson, 2009)

Its high abundance in the brain plays an essential role during the development of the nervous system of the mouse by acting as a co-repressor on the thyroid hormone receptor, but it also regulates the RORα (retinoic acid receptor-related orphan receptor α) mediated transcriptional activation of some genes, important for the cerebellar development (Cichon et al., 1998; Malloy et al., 2009; Cachon-Gonzalez et al., 1999; Potter et al., 2001; UniProt Consortium, 2012; Moraitis et al., 2002). This repressor activity can be found in the absence of ligand hormones, where the hairless protein associates with histone deactylases in so- called MAD bodies (matrix

associated deacetylase bodies) within the nucleus in order to inhibit transcription (Potter et al., 2001).

However, expression of the *hairless* gene is not only high in the brain, but also in the skin, where it is not dependent on thyroid hormone concentrations (Thompson, 1996). In the hair follicle, HR resides mainly within the ORS (including the bulge region) where its expression can be first detected in the anagen-catagen transition and remains upregulated until the next mid-anagen when the new bulb formation finally is completed and the HR level decreases to an undetectable amount (Benavides et al., 2009; Beaudoin et al., 2005). Beaudoin et al (Beaudoin et al., 2005) showed, that this expression pattern is correlated to the expression of the WNT inhibitor WISE (WNT modulator in surface ectoderm) in an inverse manner, meaning that WISE is highly expressed in bulge and bulb in late anagen when *HR* mRNA is undetectable and vice versa in telogen, which suggests a repressor function of hairless on WISE in order to enable hair follicle regeneration (Beaudoin et al., 2005; O'Shaughnessy et al., 2004). This highlights the importance of the regulatory function of HR during the hair follicle cycling but not during the follicle morphogenesis in the embryo where WNT inhibitors are redundant. Moreover, this explains why the *hairless* gene-associated alopecia patients are born with normal hair, and why their hair follicle cycling cannot be reinitiated after the ritual shedding. The mutated *hairless* gene is unable to downregulate the WNT signalling which represses WISE protein expression and thus, leads to a constant inhibition of hair follicle regeneration (Beaudoin et al., 2005).

Mutations in the *VDR* (*Vitamin D receptor*) gene have also been reported to cause an alopecia phenotype comparable to the one caused by *hairless* mutations. However, *VDR* mutations also always lead to bone-affecting Vitamin D resistant rickets, which has not been detected in patients with *hairless* mutations so far (Miller et al., 2001; Malloy and Feldman, 2011). This phenocopy can be explained by the default of the HR co-repressor action on the VDR activity, which is normally a negative transcription regulator of several genes (Malloy and Feldman, 2011).

Only very recently, a study in hair-poor mice with mutations in *U2HR* that leads to an overexpression of HR, suggested the importance of HR in the formation of the inner root sheet. The group of researchers observed that *Dlx3*, a gene important for the positive control of IRS keratin expression, was downregulated in these mice, resulting in an altered IRS (Kim et al., 2012).

By screening our families, two mutations in the *hairless* gene could be found: the nonsense mutation c.2818C>T (p.Arg940X) in exon 14 of family AP4 and the one base pair deletion c.2123delA (p.Lys708Argfs*194) in coding exon eight of family AP5. The stop codon producing p.R940X mutation has already been reported in 2007 by Kim et al (Kim et al., 2007). They proposed that this mutation at the CpG dinucleotide was produced by the spontaneous deamination of the affected cytosine to adenosine. Moreover, they highlight the fact that premature abortion of the protein could be recognized during the translation and result in a cytoplasmatic mRNA decay (Kim et al., 2007; Maquat, 1996). If the protein were translated, this would still result in an altered protein function, since the JmjC domain is missing (see Fig.4.1), which was previously shown to be required for adequate subcellular localization and repressor action of HR (Mi et al., 2011).

The deletion c.2123delA in patients of family AP5 is located before the VDR interacting domain and results in a frameshift with a premature protein truncation 194 amino acids after the first aa change. Since the aa composition after this deletion is also changed, it can be assumed that the folding may be influenced due to the different features of the new protein. The functional entities, like the repressor domains 2 and 3, the VDR interaction domain and the JmjC domain are also lost. A possible gain of function of the protein or a possible degradation in the cell remain elusive and have to be investigated.

In families AP1 and 3, mutations in the *P2RY5* gene were detected. This gene has been reported in 2008 by Pasternack et al (Pasternack et al., 2008) and Shimomura et al.(Shimomura et al., 2008) to be associated with autosomal recessive hypotrichosis and woolly hair. Hypotrichosis is characterized by sparse and short hair that can either be present in a more generalized form, like the Marie Unna type, or localized to the scalp (including eyebrows and eyelashes), called ‘localized hereditary hypotrichosis’ (LAH). Three types of LAH are known so far that are caused by mutations in the *DSG4* (LAH1), *LIPH* (LAH2) and *LPAR6/P2RY5* (LAH3) genes respectively (Shimomura et al., 2008; Kazantseva et al., 2006; Kljuic et al., 2003; Pasternack et al., 2008; Wen et al., 2009; Suga et al., 2011). While Marie Unna hypotrichosis produces very stiff-standing kinky hair, the localized form presents weaker, more fragile hair shafts (Suga et al., 2011). Moreover, LAH1 includes small papules on the scalp and hair with differing diameters from the follicle to the hair tip, resembling a wire with nodes present in a certain interval. In contrast, LAH2 and 3

have smoother hair shafts and no cysts are found on the scalp (Shimomura et al., 2008). The latter two forms can also occur in combination with woolly hair and indistinguishably have the same clinical characteristics (Petukhova et al., 2009; Azhar et al., 2012). The hair density of patients with woolly hair can vary from dense to sparse; hair filaments are extremely fine and curly and are reported to break very easily (Azhar et al., 2012). Compared to normal hair, the woolly hair is shed after only a short period of growth (Shimomura et al., 2008). Beside the isolated form, this hair shaft disease is a concomitant phenotype of the autosomal recessive Naxos disease or Carvajal syndrome. However, cardiomyopathies and palmoplantar hyperkeratosis are further diagnostic criteria of these latter diseases, which are caused by mutations in the plakoglobin gene (Naxos disease) and the desmoplakin gene (Carvajal disease) (McKoy et al., 2000; Norgett et al., 2000; Coonar et al., 1998).

Patients of Family AP1 and 3 did not show any other abnormalities other than their hair growth deficiency. While patients of family AP1 displayed the woolly hair phenotype, hypotrichosis was prevalent in family AP3, but with a varying degree of hair density among the affected. After linking both families to the LAH3 locus on chromosome 13q14.2, a homozygous tandem duplication of four base pairs, c.64_67dupTGCA, was detected in the *LPAR6/P2RY5* gene in hypotrichosis patients of family AP3. Family AP1 showed to be homozygous for the c.565G>A transition in the same gene in the left branch of the family pedigree, while in the right branch two heterozygous mutations, c.565G>A and c.188A>T, are supposed to result in a compound heterozygous disease inheritance pattern.

P2RY5 as the gene for LAH3 was published in 2008 simultaneously by two groups (Shimomura et al., 2008; Pasternack et al., 2008). Consisting of a single 1035bp long exon, it is inversely located within the biggest intron of the *RB1* gene, encoding for a 344 aa large (~30kDa) and seven transmembrane domains containing G-Protein coupled receptor protein called P2Y5 (Shimomura et al., 2008; Pasternack et al., 2008; Kent et al., 2002; UniProt Consortium, 2012). Expression studies showed that the protein is located within the Huxley's and Henle's layer of the IRS. As mentioned in the introduction, the IRS is essential for the shaping and anchoring of the hair filament (Joshi RS, 2011; Shimomura et al., 2008). Hair follicles from patients with mutations in the *P2RY5* gene lack components of the root sheet and the bulbar area is abnormally bended (Shimomura et al., 2008). The reason for the same disease phenotype in patients with *LIPH* and *P2RY5* mutations is the fact that they share the same signalling pathway. *LIPH* encodes for the Lipase H enzyme that hydrolyses

phosphatidic acid to 2-acyl-lipophosphatidic acid, a lipid mediator important, for instance, for cellular proliferation and muscle contraction by interacting with G-protein coupled receptors (Kazantseva et al., 2006; Yanagida et al., 2009). This lipophosphatidic acid further interacts with the P2Y5 (or LPA6) receptor that was proven to activate the $G_{13/12}$-Rho signalling pathway in cell culture (Yanagida et al., 2009). However, Shinkuma et al (Shinkuma et al., 2010) postulated that, based on the paper of Ohtsu et al (Ohtsu et al., 2006), the LPA-mediated receptor activation promotes via ADAMs ectodermal shedding of proteins located on the cell surface, such as EGF (epidermal growth factor), HB-EGF (heparin-binding EGF-like growth factor) and TGFα, which are supposed to play a role in the regulatory pathway of IRS cell proliferation and differentiation.

However, unlike in *HR* mutations, not the entire body hair is affected by the abnormal hair structure and deficiency in growth. Besides the same signalling pathway, the homology between LPA receptors 4, 5, and 6 is extremely high, but unlike LPA receptors 1, 2, 3, and 6, the LPA4 and LPA5 receptors are not expressed in the hair follicle of the scalp (Pasternack et al., 2008). Thus, Pasternack et al (Pasternack et al., 2008) argued that the LPA signal transduction is inhibited in P2Y5 deficient hair follicles on the scalp, whereas the expression of LPA5 in, for instance, the eyebrow follicle is able to compensate the loss of LPA6 function. In general, for the remaining body hair it is suggested, that deficient P2Y5-associated LPA signal transduction, as a result of *LPAR6* loss-of-function mutations, is supposed to also be carried out by either LPA5 or LPA4 (Pasternack et al., 2008).

However, even though highly consanguineous, in family AP1 we were initially not able to link the disease to any of the three LAH loci, since no overlapping homozygous regions nor areas with the same haplotype between family members of the right and left branch (AP1-12 and AP1-4) could be found. Only after including individual AP1-2 (left branch) in our autozygosity mapping approach, the LAH3 locus turned out to be a candidate disease locus for the left branch of the family. Surprisingly, microsatellite marker analysis of this region furthermore gave evidence for a compound heterozygous inheritance in the right branch, which was confirmed by Sanger sequencing. Both mutations have already been reported by Shimomura et al (Shimomura et al., 2008) and Azeem et al (Azeem et al., 2008); c.188A>T was also detected in another family by Kurban et al (Kurban et al., 2012). The latter missense mutation leads to the substitution of the negatively-charged asparagin acid residue at position 63 with an apolar valine, while the glutamine acid at position 188

is substituted by the positively-charged lysine (c.565G>A). Since both mutations reside within transmembrane domains (p.D63V in TM2 and p.E189K in TM5) and lead to the removal of the initially negatively-charged side chains, they were considered to interfere with the protein folding. Both mutations were reported for autosomal recessive woolly hair, which would be consistent with the phenotype seen on the pictures of patient AP1-8. Due to the possibility of the reported phenotypic variability caused by the same mutation in a family (Pasternack et al., 2009; Azhar et al., 2012), it cannot be assumed that the hair of the affected with the homozygous c.565G>A mutation in the left branch also has the same phenotypic appearance like the patients reported for this mutation by Shimomura. This is supported by the families screened by Azeem et al (Azeem et al., 2008) that were also homozygous for the same *LPAR6* mutations we and Shimomura found, but were all affected from autosomal recessive hypotrichosis and not woolly hair. It is possible, that the c.188A>T transition was initially inherited by individual II:4, but in order to confirm this DNA of all remaining family members would be needed to exclude the pedigree founders, individuals I:1 and I:2, as carrier of both alleles.

More interesting, however, is the fact that even though we screened a highly consanguineous family for which we expected an identical-by-decent mutation, we were not able to detect autozygous regions in the 250K SNP array, but rather a homozygous mutation in one branch as well as two different heterozygous mutations that segregated in the other branch. This highlights the fact that although both diseases, autosomal recessive woolly hair and localized autosomal recessive hypotrichosis, are reported to be very rare diseases, mutations in the responsible genes in the Pakistani population is obviously not that rare. Moreover, this finding points out once more (see also (Shimomura, 2012; Petukhova et al., 2009)) that one should not rely on the decreased genetic heterogeneity in consanguineous families and that compound heterozygosity should always be taken into consideration when screening such families.

Since both mutations have already been reported in Pakistani families, they are likely to be founder mutations. The Pakistani origin of these families strongly supports this assumption and if it would be possible to conduct marker analysis for the very close region around the *LPAR6* gene among all the affected from the different families, we would be able to make a statement about their relatedness. This can also be investigated for mutation p.R940X found in family AP4 and the family from Kim et al (Kim et al., 2007).

In the hypotrichosis family AP3, the homozygous frameshift causing c.64_67dupTGCA duplication located in the very beginning of the transmembrane domain 1 encoding region of *P2RY5* leads to an altered aa sequence with a premature stop codon 28 aa's after the first aa mismatch. Again, like discussed for the frameshift causing *HR* mutations, the altered sequence of this gene in this family might cause a degradation of the truncated protein, which remains to be investigated. This duplication has already been reported for two Indian girls in 2009 which were suffering from hypotrichosis with woolly hair what is consistent with the phenotype seen in our patients (Pasternack et al., 2009).

The hair phenotype of the last family, AP2, resembles the clinical picture of hypotrichosis, including sparse and lustreless hair that is progressively shed. However, in this case it occurs together with palmoplantar hyperkeratosis. Although extensive literature research has been conducted, an isolated form of these skin-affecting symptoms has obviously not been reported so far. Woolly hair can come along with a stronger form of hyperkeratosis in Naxos disease (McKoy et al., 2000; Coonar et al., 1998; Peirone et al., 2005). Our patients, however, do not suffer from heart diseases, which would be the case in Naxos disease. Also, the haplotypes of the affected at 17q21.2, the locus were the plakoglobin encoding *JUP* gene is located, have nothing in common. Both phenotypic appearances have also been reported in combination with forms of ectodermal dysplasia (Akhyani and Kiavash, 2007; Alves et al., 1981), but the multi-syndromal disease itself was much more severe than the isolated hair/skin phenotype in our patients, including neurological problems, abnormal bone morphology, teeth and eye problems, and sweat gland defects. In the genome-wide scan using the 250K NSP SNP array from Affymetrix, an autozygous region that segregated among the affected family members was found on chromosome 1, defined by a 4Mb long region between 1q21.3 up to 1q23.1. No hair diseases have been linked to this locus so far. Although the consanguinity in this family is not obvious from the pedigree, such prominent homozygous stretches like those found in the entire genome of the individuals of this family (see. Fig. 3.5) are unusual for unrelated families. The segregation of this locus among the family members was confirmed with polymorphic microsatellite markers D1S203 and D1S205 and a multipoint LOD score of 2,1 was calculated for a recessive model. This result suggests that a linkage for this locus is a 100 times more likely than no linkage. However, in order to not rule out the possibility for a compound

heterozygosity, the common heterozygous haplotypes (1q23.1-1q24.2, 8p23.3-8p23.2, and 11p15.5-p15.1) were also screened for known disease loci, but none of them linked to a reported alopecia/hypotrichosis/hyperkeratosis locus.

One other difficult aspect in this family is the question, whether the individual AP2-3 is actually affected by alopecia, whose re-growing hair seems to be much denser compared to the hair of the other affected; this, however, could be due to a variable phenotypic penetrance like the one reported for *LIPH* and *LPAR6* mutations. The candidate gene approach for *LMNA* was not successful and Sanger sequencing of all genes within the homozygous or possible compound heterozygous loci would be rather expensive and time-consuming. In this case, whole exome sequencing could be the next step after achieving the ethic approval for this family. This rather new technique allows researchers to screen all gene-encoding regions in the genome for mutations at once and is rather cost-effective compared to the single gene sequencing approach with the Sanger method (Guo et al., 2012). By then specifically filtering the region of interest and varying several parameters, like filtering SNPs, special types of mutation or mutations detected in the 1000genome project, one is able to find the underlying mutation in the expected disease locus quite easily if no candidate gene can be found in the candidate gene approach. Moreover, this method allows for the screening of all reported disease genes at once in order to rule them out for being the disease-causing gene. In case a mutation in a new gene is found, expression and functional studies could follow to determine the localization of the protein in the hair and in the cell. Furthermore, possible interaction partners remain to be revealed.

Microscopy of the deficient hair follicle and filament is another important investigation that has to be done in order to obtain information about the possible changes in follicle and filament morphology.

In conclusion, in this work especially two genes, namely the nuclear receptor co-repressor encoding *hairless* gene and the *LPAR6/P2RY5* gene, have once more been found to be mutated in individuals with congenital hair disorders. The finding of mutations in already reported disease genes is an important work in order to study the frequency of a certain disease gene which facilitates genetic counselling and screening of both, consanguineous and non-consanguineous patients. The detection of a recurrent mutation in different families can also provide information about a possible common family history (designated as ‘founder mutation’) but can over time

also develop to be specific for a certain population (Azhar et al., 2012; Shinkuma et al., 2010; Zeegers et al., 2004).

The discovery of a novel locus on chromosome 1 for a hitherto unreported hair disorder with palmoplantar hyperkeratosis obtained by this study suggests that a gene important for hair follicle morphogenesis or cycling can be found within this region. Since the hair follicle is an organ of the skin and abnormal hair growth often comes along with other skin diseases, it is very likely that these two phenotypic patterns might result from the deficiency of a single protein important for dermal signalling. Identification and characterisation of the underlying gene and its product might offer new opportunities to develop therapeutic interventions in order to finally be able to cure congenital hair disorders.

5 Abstract

Autosomal recessive hair loss is a rare disorder which is mainly found in children of consanguineous couples due to their decrease in genetic heterogeneity. The phenotypic spectrum includes universal congenital alopecia, atrichia with papular lesions, autosomal recessive hypotrichosis, and autosomal recessive woolly hair. Although the loss or gain of hair is not a life threatening disease, those affected experience psychological distress combined with a decrease in self-perception and depression due to their appearance to society.

During this work five multigenerational consanguineous Pakistani families presenting with different hair disorders were screened in order to find the underlying genetic defect. For this purpose homozygosity mapping, a reliable strategy to detect autozygous regions in the genome of consanguineous patients, was used. Microsatellite marker analysis was conducted for fine mapping of the region of interest and subsequent segregation analysis. Finally, mutations in the disease genes have been detected by Sanger sequencing.

With this approach we were able to identify three known mutations in the *P2RY5* gene in the hypotrichosis and woolly hair families, including a compound heterozygosity in one branch of a family. This finding demonstrated the limits of homozygosity mapping, for not all consanguineous patients automatically carry identical-by decent mutations.

In two universal congenital alopecia families homozygous mutations in the *hairless* gene could be revealed. While the mutation in one family had already been reported before, the c.2123delA deletion in the other family was hitherto unknown.

The fifth family did not share any known homozygous or compound heterozygous regions which overlap known disease loci. Patients of this family display sparse, short hair combined with a weak form of hyperkeratosis on the palms of hands and feet. We were able to detect a segregating 4Mb homozygous locus on chromosome 1q21.3-1q23.1 and three regions that could be interesting in terms of compound heterozygosity (1q23.1-1q24.2; 8p23.3-8p23.2 and 11p15.5-11p15.1). Further studies, such as Next generation sequencing will help to reveal the underlying disease gene and thereby will add knowledge to the understanding of the pathomechanism of the disease.

6 Zusammenfassung

Autosomal rezessiver Haarausfall ist eine seltene Krankheit, die häufig bei Familien mit konsanguinem Hintergrund zu finden ist. Zum phänotypischen Spektrum zählen unter anderem erblich bedingte universelle Alopezie, Atrichie mit papulösen Läsionen, autosomal rezessive Hypotrichose und autosomal rezessives Woolly Hair. Obwohl der Verlust von Haaren keine lebensbedrohende Krankheit ist, wirkt er sich dennoch negativ auf die Psyche der Betroffenen auf Grund der Reaktionen der Umwelt auf das veränderte Erscheinungsbild aus, was zu einer Schwächung des Selbstbewusstseins und folglich zu Depressionen führen kann.

Ziel dieser Arbeit war die Untersuchung von 5 konsanguinen Pakistanischen Familien mit Alopezie. Die dabei angewandte Homozygotie Kartierung ermöglichte den Nachweis autozygoter Bereiche im Patientengenom, in denen die Lokalisation der jeweiligen merkmalsbedingende Gene vermutet wurde. Zur genaueren Bestimmung der Grenzen dieser Bereiche und zur Überprüfung der Segregation in der Familie, wurden Mikrosatellitenanalysen durchgeführt. Mittels Sanger Sequenzierung wurden die Kandidatengene letztendlich überprüft und Mutationen dadurch identifiziert.

Mit diesem methodischen Ansatz war es möglich, bei den beiden Hypotrichose und Woolly Hair Familien drei bekannte Mutationen im *P2RY5* Gen zu detektieren. In einer dieser Familie konnte eine Compound-Heterozygotie als kausale Ursache für den beschriebenen Phänotyp nachgewiesen werden. Dieses Ergebnis zeigt einmal mehr, dass selbst bei eindeutig vorliegender Konsanguinität alle formalgenetischen Möglichkeiten in Betracht zu ziehen sind.

In den beiden von universeller Alopezie betroffenen Familien konnten jeweils homozygote Mutationen im *hairless* Gen detektiert werden. Während die Mutation c.2818C>T in einer Familie bereits publiziert wurde, ist die Deletion c.2123delA in erstmals im Rahmen dieser Arbeit gefunden worden.

In der fünften Familie konnten keine homozygoten oder mögliche compound heterozygoten Bereiche, die mit bekannten Krankheitsloci überlappen, identifiziert werden. Patienten aus dieser Familie weisen nur vereinzelte und dünne Haare auf, die in Kombination mit einer leichten Hyperkeratose auf Handflächen und Fußsohlen auftreten. Die SNP-Analysen ergaben einen segregierenden 4Mb großen Homozygotie-Bereich auf Chromosom 1q21.3-1q23.1 und zusätzlich drei Kandiatenregionen für eine mögliche Compound-Heterozygotie (1q23.1-1q24.2; 8p23.3-8p23.2 und 11p15.5-11p15.1). Weitere Studien und Experimente, wie z.B.

Next Generation Sequencing, zur Identifikation des Krankheitsgens und der Charakterisierung des daraus resultierenden Proteins sind in Planung.

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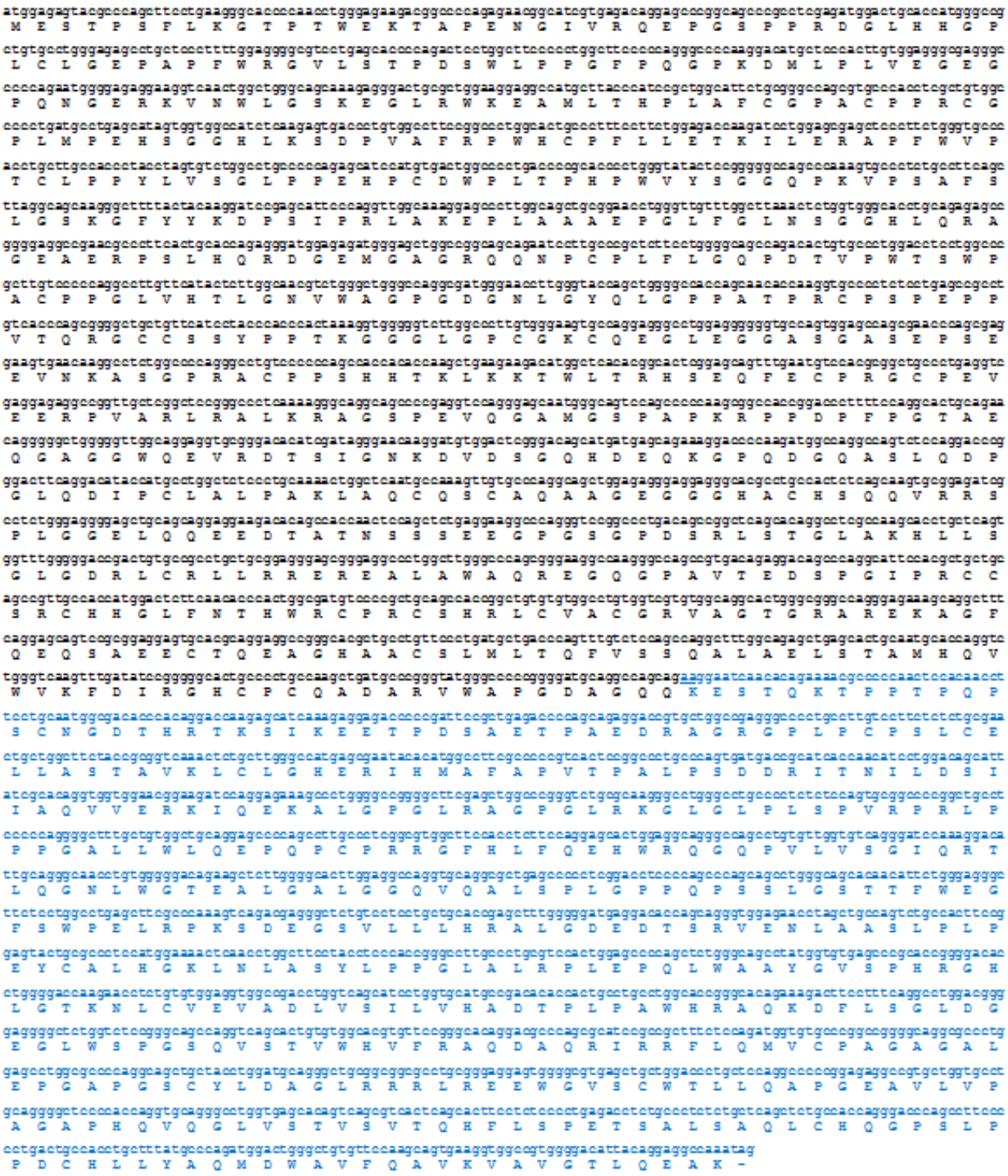
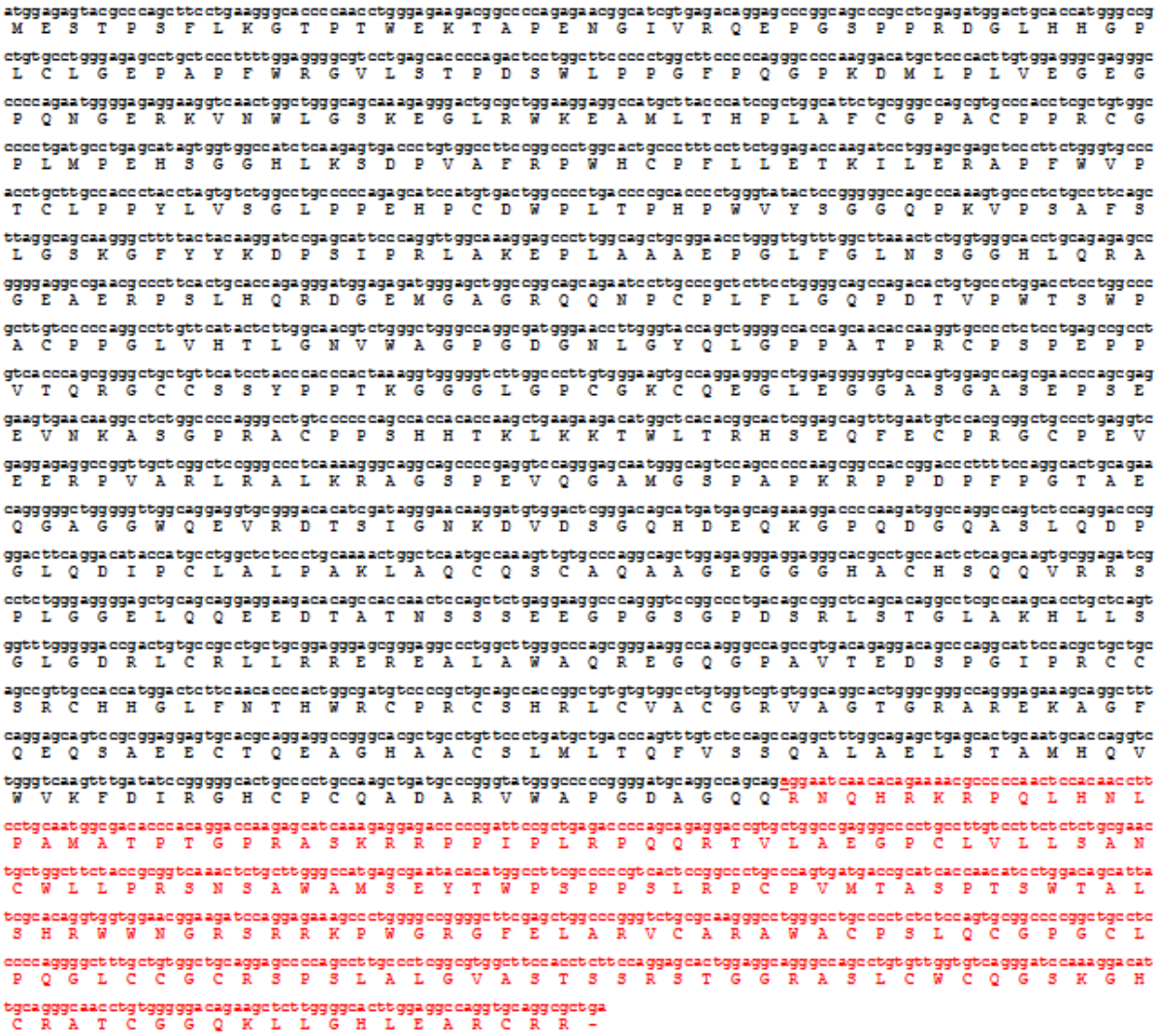
8 Appendix

HR WT



Supplementary scheme 1: wildtype *hairless* cDNA sequence with the corresponding amino acid sequence. The first two adenosines of exon 8, of which one is deleted in patients of family AP5, are underlined in blue; the sequence that will be affected by the frameshift is highlighted in blue as well. Aa translation was generated using the ExPASy translation tool (http://web.expasy.org/translate/) (Gasteiger et al., 2003)

HR p.Lys708Argfs*194



Supplementary scheme 2: Altered *hairless* frame of the cDNA and aa sequence after the deletion of one adenosine in the beginning of exon 8 (highlighted in red) in alopecia patients of family AP5. Compared to supplementary scheme 1, it can be seen, that a premature stop codon is found 194 aa's after the last aa, which coincides with the wt frame. Aa translation was generated using the ExPASy translation tool (http://web.expasy.org/translate/) (Gasteiger et al., 2003)