Identification and characterization of genes for skin and hair disorders

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List of abbreviations

AD	autosomal dominant
AR	autosomal recessive
COLED	Cole disease
DNA	Deoxyribonucleic acid
EV	epidermodysplasia verruciformis
ED	Ectodermal dysplasia
ES	Exome sequencing
HF	Hair follicle
IF	intermediate filament
OMIM	Online Mendelian Inheritance in Man
PPPK	punctate palmoplantar keratoderma
mRNA	messenger Ribonucleic acid
RT-qPCR	reverse transcription quantitative real-time polymerase chain reaction

1. Abstract

The skin, as the outermost organ of the human body along with its appendages (e.g. hair), possesses a remarkable capacity for self-regeneration, as well as social and functional significance, distinguishing it from many other tissues. Skin and hair disorders comprise conditions in which affected individuals display one or more of the following features: dyspigmentation (e.g. Cole disease, COLED); abnormal skin structure (e.g. epidermodysplasia verruciformis, EV); or abnormal hair structure and growth (e.g. monilethrix). Skin and hair disorders develop as a consequence of genetic and/or environmental factors. In some cases, genetic influence manifests as a direct causative factor, as exemplified by monogenic disorders such as monilethrix or COLED. While significant progress has been made in identifying causal genes for several skin and hair disorders, others remain unexplained. Further research is required to elucidate the underlying molecular genetic mechanisms, which can improve diagnostic accuracy and facilitate the development of therapeutic strategies.

Therefore, the aim of the present thesis was to genetically elucidate rare monogenic skin and hair disorders by identifying novel pathogenic variants and/or genes, and their underlying pathomechanisms.

In all three studies included in the present thesis, we performed Sanger and/or exome sequencing (ES) in DNA from patients affected by skin and hair disorders, namely, COLED, EV, and monilethrix. We identified novel pathogenic variants in genes previously associated with COLED and EV and, for the first time unraveled a novel type I keratin gene as the genetic basis of monilethrix, that is *KRT31*. In detail, we identified a novel pathogenic variant in *ENPP1* in the somatomedin-B-like 2 (SMB2) domain in a family with COLED. In a consanguineous family with four individuals affected by EV, we identified a novel pathogenic variant in *TMC8* leading to abnormal splicing. And we found a nonsense variant in *KRT31* as a novel cause of monilethrix in six individuals from four unrelated families. We performed cell culture experiments, e.g. immunoblotting, immunofluorescence and reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR), to confirm the pathogenicity of the variant.

With these results, we have expanded knowledge on genetic hair and skin disorders. In particular, we have: i) broadened the genetic spectrum of *ENPP1*-associated COLED; ii) expanded the mutation spectrum of EV by identification of a *TMC8* variant; and iii) first

unravelled a pathogenic variant in *KRT31* as cause of autosomal dominant monilethrix.

2. Introduction and aims with references

2.1 Skin and hair follicle anatomy, function, morphogenesis

Skin is the largest organ of the body, accounting for almost 15% of the total body weight in adults. It performs several functions, including acting as a barrier to restrict UV light, immune surveillance, mechanical stimuli, the perception of touch, temperature, pain, and the regulation of temperature and hydration (Boyce, 2001). With its complex and vital function, the skin constitutes the integumentary system with appendages (e.g. hair follicle (HF), nail, sweat/sebaceous glands) (Kanitakis, 2002; Vig et al., 2017). The skin is composed of three distinct layers (Figure. 1, left), from the upper surface to the bottom surface, named: i) epidermis (including appendages, pilosebaceous follicles and sweat glands); ii) dermis, which was separated from epidermis by the dermal-epidermal junction; and iii) hypodermis (Kanitakis, 2002). From the perspective of embryology, the epidermis and its appendages are of ectodermal origin, whereas the dermis and hypodermis are of mesodermal origin (McKee, 1996; Murphy, 1997; Cribier and Grosshans, 2002). It is interesting to note that the palms and soles are distinguished by hairless skin, in contrast to the rest of the body, which is predominantly covered by hair-bearing skin (Kanitakis, 2002).

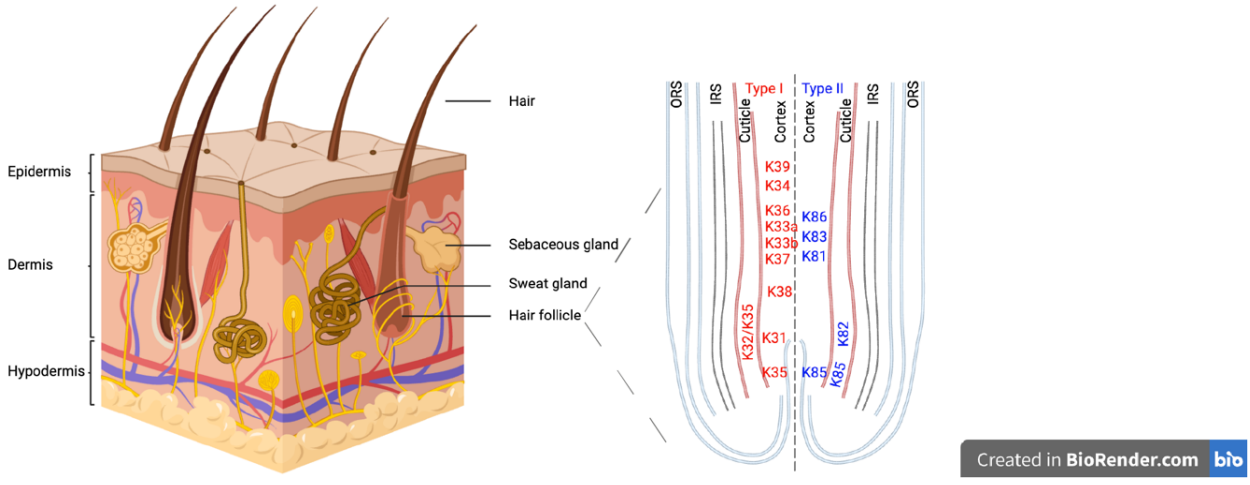

Fig. 1: The image on the left depicts the anatomy of the skin and hair, while the image on the right illustrates the keratin that constitutes the hair shaft. (Created in https://BioRender.com)

Hair is one of the skin appendages which originate from the ectoderm whereas its proliferation and morphogenesis relays on the signal interactions between the epidermis and

dermis (Fuchs, 2007). Fuchs demonstrated that HF results in the production of terminal hair, which extends into the deep dermis and even the subcutis. The hair is composed of terminally differentiated keratinocytes, which are compacted into a fiber with exceptional tensile strength known as the hair shaft. During the hair growth cycle, which is known as the anagen, catagen, and telogen stages, keratins are synthesized by the keratinocytes and play an essential role in the formation of hair shafts. It is worth of note that hair keratins exhibit a particularly complex expression pattern in the hair-forming compartment of the follicles (Figure. 1, right) (Langbein et al., 2007). In general, two types of hair keratins can be identified, which are designated as Type I keratin (for example, keratin 31 to 40) and Type II keratin (for example, keratin 81 to 86). Hair keratins include type I keratins, which form intermediate filaments (IF) with type II keratins and are involved in cell mobility, cell proliferation, cytodifferentiation and cell cycle progression (Foisner, 1997; Magin et al., 2007; Moll et al., 2008; Oriolo et al., 2007). Therefore, the typical interactive combination of the keratins is a key feature for normal hair morphology.

2.2 Genetic of skin and hair disorders

The number of genetic skin disorders is extremely high, and their aetiologies and clinical features vary a lot. A previous study reported that more than thousands genetic skin disorders have been documented, but the distinct molecular basis has been identified for less than 600 of them while the underlying genetic abnormalities for the remaining disorders have yet to be determined (Feramisco et al., 2009; Moss, 2009). Given the complex nature of the skin as the body's outermost organ, any malfunction or disruption to its optimal functioning can lead to a range of adverse conditions affecting skin and hair. These can include conditions such as rash dermatitis and abnormal hair growth, which may have a significant impact on an individual's quality of life. In severe cases, the disruption of skin can even pose a threat to life (Schneider et al., 2009).

Skin and hair disorders have highly diverse genetic backgrounds. Monogenic inherited disorders have pathogenic variants in a single gene whereas multifactorial disorders involve variants in multiple genes and environmental factors. In general, the morphogenesis and proliferation of skin and hair rely on the precise regulation of essential signalling pathways (e.g. insulin signalling pathway) and properly structured proteins (e.g. keratin).

Therefore, any abnormal variants affecting these pathways or proteins can potentially lead to disorders.

2.2.1 Cole disease

Cole disease (COLED, MIM 615522) is a genodermatosis characterized by dyspigmentation and punctate palmoplantar keratoderma (PPPK). Classical features of COLED include cutaneous dyspigmentation, commonly hypopigmented macules of varying sizes and rarely hyperpigmented macules predominantly located on the extremities, PPPK and infrequently ectopic calcifications. Pathogenic variants in *ENPP1*, which encodes ectonucleotide pyrophosphatase/phosphodiesterase 1, cause COLED, which can be inherited in either an autosomal dominant (AD) or autosomal recessive (AR) manner (Chourabi et al., 2018; Eytan et al., 2013; Schlipf et al., 2016). Of particular importance is the somatomedin-B-like 2 domain of *ENPP1*, which plays a role in the regulation of epidermal pigmentation and differentiation (Eytan et al., 2013). To date, only 13 families have been reported in the literature and most of the affected individuals show pathogenic variants in *ENPP1*.

2.2.2 Epidermodysplasia verruciformis

Epidermodysplasia verruciformis (EV, MIM 618231, 226400 and 618267) is a rare autosomal recessive skin disorder. However, an X-linked recessive inheritance of EV was reported in a well-characterised family, where only males were affected (Androphy et al., 1985). EV is characterized by persistent disseminated flat warts and pityriasis versicolor-like lesions associated with abnormal susceptibility to the human beta papillomavirus (Orth, 2006). Patients with EV start to present with symptoms in infancy or childhood. Their risk for carcinoma is quite high, especially in sun-exposed areas. To date, pathogenic variants in either *TMC6* (*EVER1*) or *TMC8* (*EVER2*) have been identified in more than half of the reported patients with typical EV (Imahorn et al., 2017; Ramoz et al., 2002). Recently, one study indicated that biallelic pathogenic variants in *CIB1* underlies the molecular basis of EV (de Jong et al., 2018). It is worthy of note that CIB1 can form a complex with TMC6 and TMC8 to act as restriction factor against the human beta papillomavirus.

2.2.3 Monilethrix

Monilethrix (MIM 158000 and 607903) is a rare hereditary disorder of the hair shaft structure that affect male and female individuals equally. Previous research has identified both AD and AR modes of inheritance. The typical monilethrix is characterized by dystrophic alopecia of the scalp, which manifests predominantly at the occiput. Individuals with monilethrix are generally born with normal hair, but structural hair changes usually emerge within the first few months of life (Sinclair, 2016). The condition may persist throughout life, although hair structure can improve during adolescence or occasionally during pregnancy. The severity of monilethrix varies significantly both among and within families, ranging from mild scalp thinning to near-total baldness with a characteristic “stubble field” appearance (Alexander and Grant, 1958).

Under microscopic examination, affected hairs display spindle-shaped variations in thickness, resulting from elliptical nodules with normal hair shaft diameter alternating with dystrophic constrictions at regular intervals. These constrictions give the hair a beaded appearance and make it prone to breakage at these points (Ito et al., 1990). Additional clinical features include follicular hyperkeratosis, predominantly on the occiput and neck, observed in up to 90% of cases, as well as nail abnormalities such as koilonychia and platonychia reported in many families (Korge et al., 1998).

Monilethrix was initially documented in 1880; however, the first pathogenic variant was identified only a century later in *KRT86* (Smith, 1880; Winter et al., 1997a). Since then, several pathogenic variants were identified in *KRT86*, while only a few were found in *KRT81*, *KRT83* and *DSG4* (Kljuic et al., 2003; Schaffer et al., 2006; Shimomura et al., 2006; Van Steensel, 2005; Winter et al., 1997b; Zlotogorski et al., 2006). The three keratin genes code for type II keratins and are responsible for AD inheritance manner, while *DSG4* is responsible of the AR monilethrix. Prior to our study, genetic associations between type I keratins and monilethrix remained undefined.

2.3 Research Aims

The work presented in the present thesis aims to: i) identify novel pathogenic variants associated with skin and hair disorders; ii) unravel novel genes linked to hair abnormalities; and iii) deepen the understanding of genotype-phenotype correlations of pathogenic variants implicated in skin and hair disorders, as well as achieving a more comprehensive

understanding of the pathomechanisms underlying these disorders. By characterising both known and novel genes involved in skin and hair development, our research aims to make further understanding to the biology of skin and hair. Consequently, our work will improve the molecular diagnostic yield for rare ectodermal phenotypes of skin and hair tissues.

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3. Publications

3.1 Publication 1:

Xiong X, Cesarato N, Gossmann Y, Wehner M, Kumar S, Thiele H, Demuth S, Oji V, Geyer M, Hamm H, Basmanav FB, Betz RC. A nonsense variant in *KRT31* is associated with autosomal dominant monilethrix. Br J Dermatol. 2024; 191(6):979-987 https://doi.org/10.1093/bjd/ljae298

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A nonsense variant in *KRT31* is associated with autosomal dominant monilethrix

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Abstract

Background Monilethrix is a rare hereditary hair disorder that is characterized by a beaded hair shaft structure and increased hair fragility. Patients may also present with keratosis pilaris and nail changes. Research has identified three genes responsible for autosomal dominant monilethrix (*KRT81*, *KRT83*, *KRT86*) and one responsible for the autosomal recessive form (*DSG4*).

Objectives To investigate the genetic basis of autosomal dominant monilethrix in families with no pathogenic variants in any of the known monilethrix genes, and to understand the mechanistic basis of variant pathogenicity using a cellular model.

Methods Nine affected individuals from four unrelated families were included. A clinical diagnosis of monilethrix was assigned based on clinical examination and/or trichoscopy. Exome sequencing was performed in six individuals to identify pathogenic variants; Sanger sequencing was used for co-segregation and haplotype analyses. Cell culture experiments [immunoblotting, immunofluorescence and reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) analyses] were used to confirm variant pathogenicity, to determine the expression and subcellular localization of proteins, and to identify possible nonsense-mediated mRNA decay.

Results In six affected individuals with clinically suggested monilethrix, exome sequencing led to the identification of the nonsense variant c.1081G>T; p.(Glu361*) in *KRT31*, which was subsequently identified in other affected members of these families by Sanger sequencing. This variant led to the abolition of both the last three amino acids of the 2B subdomain and the complete C-terminal tail domain of keratin 31. Immunoblotting demonstrated that when co-expressed with its binding partner keratin 85, the truncated keratin 31 was still expressed, albeit less abundantly than the wildtype protein. Immunofluorescence revealed that p.(Glu361*) keratin 31 had an altered cytoskeletal localization and formed vesicular-like structures in the cell cytoplasm near the cell membrane. RT-qPCR analysis did not generate evidence for nonsense-mediated decay of the mutant transcript.

Conclusions This study is the first to identify pathogenic variants in *KRT31* as a cause of autosomal dominant monilethrix. This highlights the importance of hair keratin proteins in hair biology, and will increase the molecular diagnostic yield for rare ectodermal phenotypes of hair and nail tissues.

Lay summary

The structure of human hair can vary widely. Some people have straight hair, while other people have curly or 'woolly' hair. Few people have a beaded hair structure known as 'monilethrix'. This hair structure causes hair fragility, leads to hair loss and may be associated with changes in the skin and/or nails. Monilethrix is a genetic condition, which means that it is caused by changes in an affected person's DNA. These genetic changes are also called 'mutations'. So far, mutations in four genes (*KRT81*, *KRT83*, *KRT86* and *DSG4*) have been identified for monilethrix.

We investigated nine people from four unrelated families who had monilethrix. In these families, we found no pathogenic variant in any of the known monilethrix genes. We then carried out a technique that allowed us to look at all their genes. We discovered a new genetic cause for monilethrix in a gene called '*KRT31*'. The protein associated with *KRT31* helps to form structures termed intermediate filaments. They make up the skeleton of the cell and help maintain the integrity and mechanical properties of the hair, skin and nails. The changes we saw in *KRT31* altered the protein and disrupted the location of intermediate filaments.

Our study identified a new genetic cause for monilethrix. This finding expands understanding of the genes important in hair structure, skin growth and nail integrity. Our research could help improve the diagnosis and management of people with monilethrix.

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What is already known about this topic?

- Keratins and desmosomes form and connect intermediate filaments (IFs) and are thus essential in terms of cellular integrity.
- In humans, pathogenic variants in genes that encode keratins 81, 83 and 86, and desmoglein 4, cause autosomal dominant and autosomal recessive monilethrix, respectively.
- Several IF-associated keratins are associated with human genetic diseases that affect ectodermal tissues.

What does this study add?

- A pathogenic nonsense variant in *KRT31* causes autosomal dominant monilethrix.
- Keratin 31 (KRT31), encoded by *KRT31*, is located in the cytoplasm of keratinocytes and is involved in IF construction.
- Mutated KRT31 resulted in an aberrant pattern of subcellular localization.

What is the translational message?

- This study is the first to describe *KRT31*-related monilethrix.
- The inclusion of *KRT31* in diagnostic gene panels for hair, skin and nail-related disorders will improve diagnostic yield.
- This discovery will facilitate appropriate clinical management and genetic counselling for patients and families with monilethrix.

Monilethrix (MIM: 158000 and 607903) is a rare hereditary disorder of the hair shaft structure that affect male and female individuals equally. Monilethrix is characterized by a dystrophic alopecia of the scalp, which manifests predominantly at the occiput. Affected individuals typically present with normal hair at birth, with hair changes first becoming evident during the first months of life.[1] In some cases, the disorder may persist across the lifespan. Alternatively, hair structure may improve either during adolescence, or sporadically during pregnancy.[2] The monilethrix phenotype varies widely both within and between families, ranging from absent or minimal alopecia of the scalp to almost complete baldness with a 'stubble field' appearance.[2]

Microscopic examination of affected hair reveals spindle-like variations in calibre. These are caused by a series of elliptical nodules with normal hair shaft diameter and dystrophic constrictions in regular intervals, which produce a beaded appearance and a tendency – at these constrictions – for the hair shaft to break.[3] Additional signs include follicular hyperkeratosis – mostly on the skin of the occiput and neck – in up to 90% of patients and nail changes, particularly koilonychia and platonychia, in many families.[4]

Previous research into monilethrix has identified both autosomal dominant and autosomal recessive modes of inheritance. The first pathogenic variant was identified in *KRT86* by Winter *et al.* in 1997.[5] Since then, most cases of monilethrix have been explained by pathogenic variants in *KRT86*, which are inherited in an autosomal dominant manner. Later research identified – and only in a few individuals – dominant pathogenic variants in the genes *KRT83* and *KRT81*.[6] Most of the known variants in these three genes are located in a mutational hotspot of the region that encodes the helix termination motif (HTM). These keratin genes code for type II keratins, which are components of the cytoskeleton and play a role in cellular mechanical stabilization.[7] An autosomal recessive form of monilethrix has also been described, involving biallelic pathogenic variants in *DSG4*, which encodes a component of the desmosome, a cell structure involved in cell–cell adhesion.[8–11] All four genes are expressed in the cortex of the hair shaft and help to stabilize the hair against physical stress.[7,12–14] The clinical signs of monilethrix are similar, irrespective of which of the four different causal genes is implicated, and thus no conclusions can be drawn concerning the responsible underlying mutation on the basis of clinical presentation alone.

Keratins comprise a large family of intermediate filament (IF)-forming proteins with a highly conserved secondary structure and tissue-specific expression.[15] All keratins have an N-terminal head domain, a central α-helical rod domain and a C-terminal tail domain.[16] The C-terminal component of the rod domain is subdomain 2B, part of which functions as an HTM.[17] Keratins are divided into two main categories: (i) type I keratins, also termed acidic keratins; and (ii) type II keratins, also termed basic keratins. Type I and type II keratins bind to create acidic-basic heterodimers, which form the IFs. These IFs are components of the cytoskeleton, and are involved in cell mobility, cell proliferation, cytodifferentiation and cell cycle progression.[18–21] In addition, the IF network binds to the desmosomes, whose composition includes desmoglein 4, and allow cell–cell interactions and a mechanical strengthening of the cells.[14]

We identified a novel molecular cause of autosomal dominant monilethrix via the identification of a stop variant in *KRT31* in nine affected individuals from four unrelated families. Functional analyses in cultured keratinocytes implicate impairments in the subcellular localization of the mutated keratin 31.

Materials and methods

Study participants

Our cohort comprised nine individuals (five female and four male patients) from four unrelated families, all of whom had presented with a suspected clinical diagnosis of monilethrix and no pathogenic variant in the known genes for monilethrix. These individuals had either been referred for genetic

counselling (to R.C.B.) or their blood/saliva samples had been sent to our laboratory for molecular genetic diagnostic analysis.

Exome and Sanger sequencing

For six of the nine individuals (three male and three female patients), the collected DNA was subjected to exome sequencing. Exome sequencing was performed at the Cologne Center for Genomics (Cologne, Germany), as described elsewhere.[22] The exome sequencing data were filtered to identify genes containing rare variants (minor allele frequency < 1%). For the remaining three individuals, Sanger sequencing was performed to screen for *KRT31* variants. Sanger sequencing was also used for exome sequencing result verification, co-segregation analysis and genotyping of single nucleotide polymorphisms for ancestry analysis, using the BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and an ABI 3100 genetic analyser (Applied Biosystems).

Cell culture and vectors

In vitro cell culture experiments were conducted using (i) human embryonic kidney (HEK) HEK293T cells (kind gift from Thomas Zillinger, Institute of Clinical Chemistry and Clinical Pharmacology, University of Bonn, Bonn, Germany) and (ii) HaCaT cell lines (as established by Boukamp *et al.*).[23] Human DYK-tagged *KRT31* was either expressed alone or together with its binding partner HA-tagged *KRT85* using a co-expression system (GenScript Biotech, Piscataway, NJ, USA), as described in detail in Appendix S1 (see Supporting Information). The *KRT31* variants p.(Glu361*) and p.(Asn366Lysfs*) were generated using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA, USA).

Immunoblotting

HEK293T cells were transfected via Lipofectamine 3000 (ThermoFisher Scientific, Waltham, MA, USA) 24 h after seeding. At 24 h post-transfection, the cells were lysed in RIPA buffer supplemented with Protease Inhibitor Cocktail (ThermoFisher Scientific) and then centrifuged at 16 000 *g* for 10 min. The supernatants were recovered and quantified via a Bradford assay. For each sample, 40 μg extracted protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis for the purpose of immunoblot analysis with anti-DYK antibody (F1804; Sigma-Aldrich, St. Louis, MO, USA) and anti-HA antibody (3724S; Cell Signaling Technology, Danvers, MA, USA). Visualization of the bands and quantitation were performed using the Chemidoc imaging system and Image Lab software (Bio-Rad Laboratories, Hercules, CA, USA). The procedure was performed in three or more replicates. Quantitation was performed via normalization of the bands to total protein content in each lane. Statistical analysis was performed with Prism (GraphPad, La Jolla, CA, USA), with a *P*-value < 0.05 denoting statistical significance.

Immunofluorescence staining

HaCaT cells were seeded on coverslips. After 24 h, the cells were transfected via Lipofectamine 3000 (ThermoFisher Scientific). At 36 h post-transfection, the cells were fixed with paraformaldehyde 3.7%, permeabilized in 0.1% Triton X-100 and blocked with bovine serum albumin supplemented with 1% normal goat serum. The cells were then incubated with anti-DYK antibody (F1804; Sigma-Aldrich), anti-HA antibody (3724S; Cell Signaling Technology) and/or anti-LAMP1 antibody (9091T; Cell Signaling Technology) for 1 h, followed by incubation for 1 h with the secondary antibodies antimouse fluorescein isothiocyanate (ab6785; Abcam, Cambridge, UK) and antirabbit Alexa Fluor 568 (A-11011; ThermoFisher Scientific). Slides were then mounted with VECTASHIELD® (Vector Laboratories, Newark, CA, USA). Images were generated using a CytoVision DM5500B microscope (Leica Biosystems, Deer Park, IL, USA) and the accompanying CytoVision 7.4 software.

Reverse transcription quantitative real-time polymerase chain reaction

Total RNA was isolated from transfected HEK293T cells with NucleoSpin RNA Plus kit (Macherey-Nagel, Düren, Germany). Total RNA (2 μg) was used for cDNA synthesis with SuperScript III Reverse Transcriptase (ThermoFisher Scientific). Relative *KRT31* expression from wildtype (WT) and mutant sequences (i.e. relative quantification) was determined by real-time polymerase chain reaction (PCR) using EvaGreen (Bio-Budget Technologies, Krefeld, Germany) and a QuantStudio 5 Real-Time PCR System (ThermoFisher Scientific) and the comparative threshold cycle (Ct) method $2^{-\Delta\Delta Ct}$ in three or more replicates. The expression of *GAPDH* and *RPL30* was used as an endogenous control. Statistical analysis was performed using Prism (GraphPad) with a statistical significance threshold of $P < 0.05$.

Results

Clinical presentation of the affected individuals

Four unrelated families from Germany were investigated. In family 1, the parents of a 15-year-old girl (IV:2) reported that she had had normal hair at birth. Diffuse scalp hair loss had then occurred in phases, with no involvement of the eyebrows or eyelashes (Figure 1a). A similar hair phenotype was observed in seven additional family members over four generations, indicating an autosomal dominant pattern of inheritance (Figure S1a; see Supporting Information). Microscopic analysis of the scalp hair of girl IV:2 revealed spindle hairs with fluctuations in calibre. Affected girl IV:2 was also reported to have had a history of brittle and fragile nails and scalp itchiness since birth. The remaining eight affected individuals presented with a similar phenotype. Detailed clinical descriptions of all nine patients are presented in Table S1 (see Supporting Information) and Figure 1.

KRT31 nonsense variant p.(Glu361*) co-segregated in four monilethrix families

After the exclusion of pathogenic variants in the known genes for monilethrix (i.e. *KRT81*, *KRT83*, *KRT86* and *DSG4*) – via examination of one individual from each of the four families – exome sequencing was done for six affected individuals

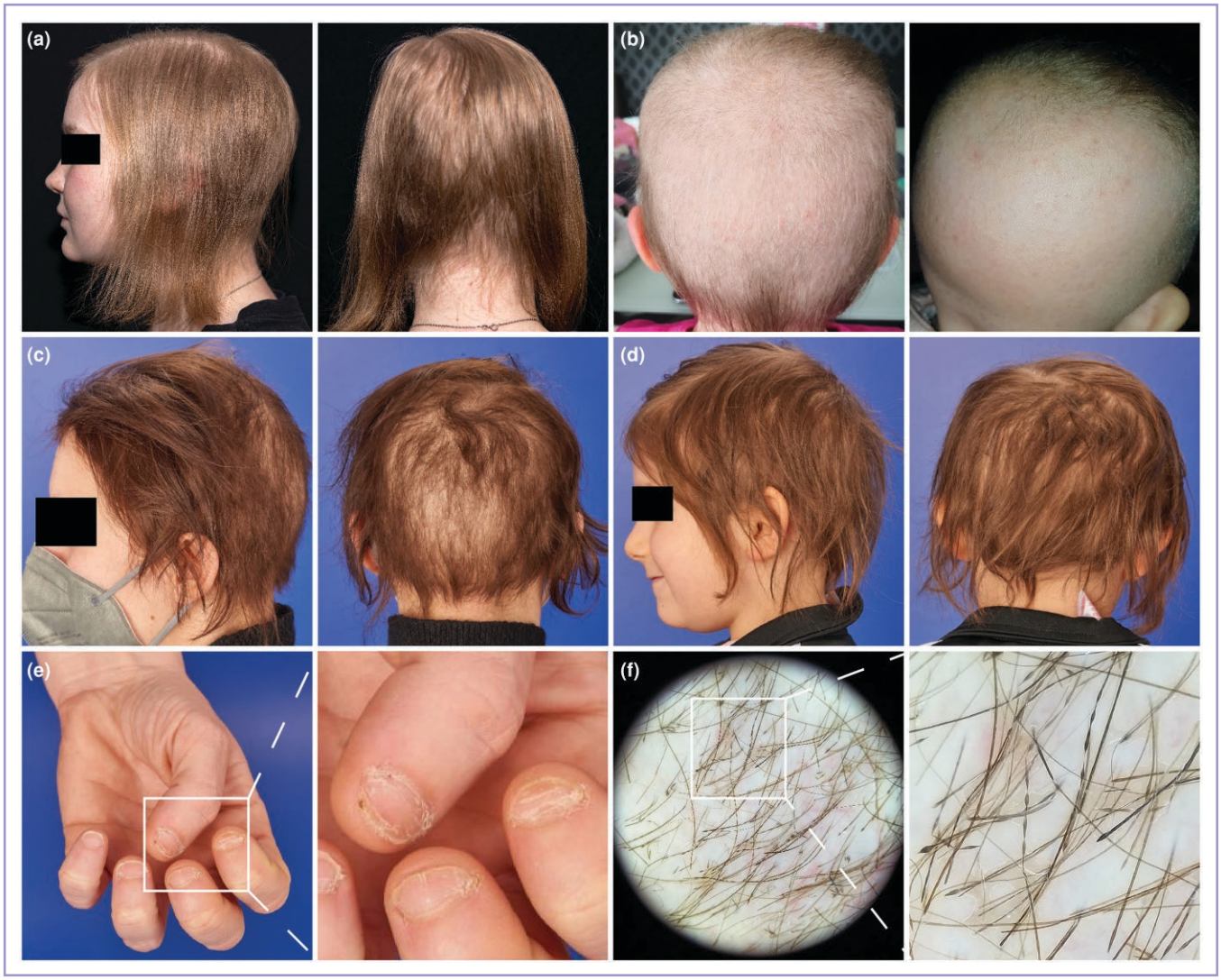

Figure 1 Clinical findings in individuals with the pathogenic *KRT31* variant. (a) Family 1 individual IV:2 has sparse, diffuse hair on the vertex and the occipital region. (b) Family 2 individual IV:1 has sparse hair and typical follicular keratoses on the skin of the scalp. (c, d) Family 3 individuals I:2 and II:1 have reduced scalp hair with accentuated alopecia in the occipital region. (e) Family 3 individual I:2 presents with platonychia and fragile nails. (f) Trichoscopic images from family 3 affected individual I:2, show the typical nodes and narrow internodes of the hair shafts.

from these four families. Potentially pathogenic variants in these individuals were then compared. All six individuals carried the heterozygous variant c.1081G>T; p.(Glu361*) in exon 6 of *KRT31* (Figure S1e), which is composed of seven coding exons. The variant is absent in gnomAD version 4.0.0 (https://gnomad.broadinstitute.org/). Sanger sequencing in three additional affected family members revealed that the variant co-segregated with the clinical picture.

To determine whether this pathogenic variant derived from a single mutational event (i.e. founder effect) or arose independently (e.g. due to the nucleotide structure), haplotype analyses were performed with six markers around the variant in all members of the four families (Appendix S2; see Supporting Information). Haplotype analyses showed that all affected individuals shared a common haplotype, suggesting that the variant p.(Glu361*) derived from a single mutational event (Figure S2; see Supporting Information).

p.(Glu361*) keratin 31 shows no difference in expression vs. the wildtype when expressed in the absence of its binding partner keratin 85

To analyse possible differences in the intracellular processing of WT and mutant keratin 31, immunoblotting analyses were performed. *KRT31* variant p.(Asn366Lysfs*) was included as a control. This is located close to the p.(Glu361*) variant, is predicted to generate a similar truncated protein p.(Asn366Lysfs*) and is annotated as benign. In previous reports, individuals with this variant showed a normal hair phenotype.[24,25]

HEK293T cells were transfected with plasmids containing *KRT31* WT and both of the aforementioned mutant sequences. As expected, mutant keratin 31 proteins had a lower molecular weight than the WT protein (Figure 2b). No difference was found between the abundance of WT keratin 31 and that of the pathogenic variant p.(Glu361*), while

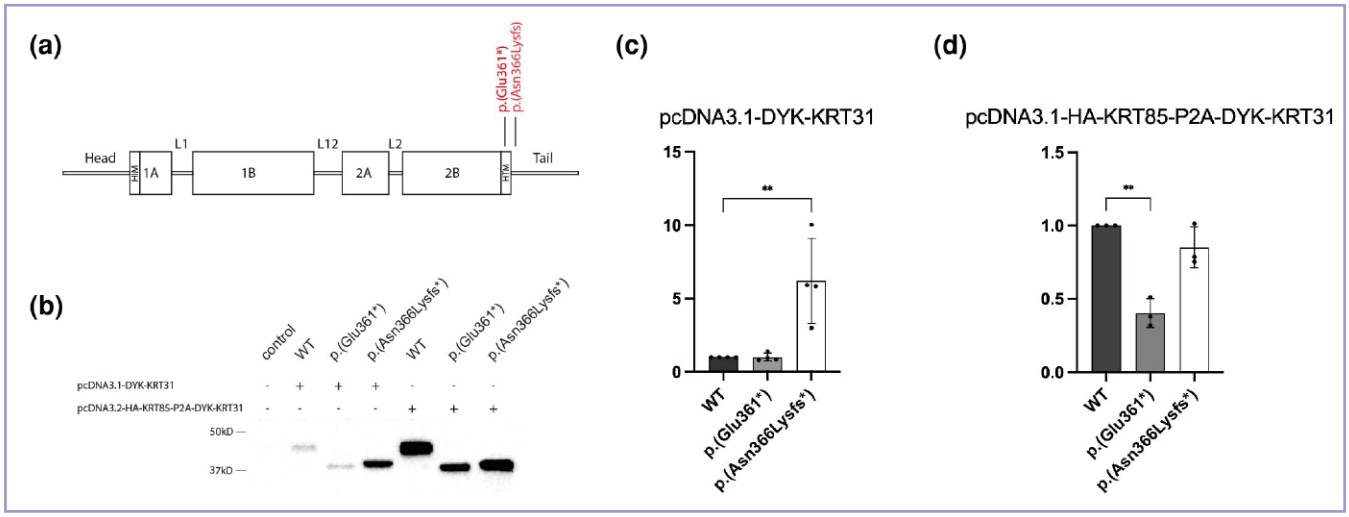

Figure 2 Keratin 31 expression and statistical analysis. (a) Schematic of the protein structure of keratin 31 and localization of the analysed variants. The p.(Glu361*) is located on the 2B domain, whereas the p.(Asn366Lysfs*) is located on the tail domain of the protein. (b) Representative immunoblotting figure of HEK293T cells transfected with the single and co-expression plasmids; antibodies against DYK-tag. (c) Relative expression of keratin 31 mutants p.(Glu361*) and p.(Asn366Lysfs*) vs. the wildtype (WT) form in the single-expression system. (d) Relative expression of the keratin 31 mutants p.(Glu361*) and p.(Asn366Lysfs*) compared to the WT form in the co-expression system. Statistical analyses were performed on data derived from three or more independent biological replicates.

the benign p.(Asn366Lysfs*) variant was significantly more abundant than both the WT keratin 31 and the p.(Glu361*) variant (Figure 2c).

To assess a possible nonsense-mediated decay (NMD) of p.(Glu361*) mRNA, we extracted RNA from transfected HEK293T cells and performed reverse transcription quantitative real-time PCR (RT-qPCR). Statistical analysis showed no difference in *KRT31* mRNA abundance between cells transfected with WT and mutant *KRT31* and therefore not supporting NMD of the mutant transcripts (Figure S3; see Supporting Information).

p.(Glu361*) keratin 31 is less abundant than the wildtype when co-expressed with keratin 85

Keratin 31 is a type I keratin; keratin 85 is its reported binding partner.[26] Therefore, we used a co-expression plasmid in which *KRT85* was separated from *KRT31* by the sequence coding for the P2A self-cleaving peptide. While the P2A peptide of the co-expression system showed a high cleaving efficiency, the uncut construct remained visible (Figure S4a; see Supporting Information).

Interestingly, in this co-expression system, the abundance of the WT protein was similar to that of the benign variant p.(Asn366Lysfs*), whereas the abundance of the monilethrix variant p.(Glu361*) was significantly less than that of the WT protein (Figure 2d). The abundance of keratin 85 was consistent across co-expression constructs containing WT keratin 31 and the two mutants (Figure S4b).

p.(Glu361*) keratin 31 creates vesicular-like structures

As the keratin network is a component of the cytoskeleton, immunofluorescence was performed to detect changes in the cellular localization of WT and mutant keratin 31 within HaCaT cells. In the single protein expression system, the WT keratin 31 and its benign variant p.(Asn366Lysfs*) formed filaments in the cytoplasm (Figure 3a). In contrast, the mutant p.(Glu361*) protein formed vesicular-like structures that mainly localized around the cell membrane. Given that no co-localization was observed with the lysosomal marker LAMP-1 (lysosomal-associated membrane protein 1), it is unlikely that these vesicles corresponded to lysosomes (Figure S5; see Supporting Information). In the co-expression system, the binding partner (keratin 85) also formed filaments in the cytoplasm, compatible with a cytoskeletal localization, and the pathogenic p.(Glu361*) variant continued to create vesicular-like structures that were mainly located around the cell membrane. This shows that the presence of keratin 85 did not prevent the formation of this aberrant presentation (Figure 3b). Furthermore, the WT keratin 31 and the benign variant p.(Asn366Lysfs*) showed strong co-localization with its binding partner keratin 85, whereas the vesicular-like structures formed by monilethrix variant p.(Glu361*), which were located in the vicinity of the cell membranes, showed impaired co-localization with keratin 85 (Figure 3b).

Discussion

To our knowledge, this study is the first to implicate *KRT31* in monilethrix. Analyses demonstrated that a pathogenic variant of *KRT31* caused an autosomal dominant form of the disorder in nine individuals from four unrelated families. While all other keratin genes implicated in monilethrix to date are located on chromosome 12 and code for type II basic keratins, *KRT31* is located on chromosome 17 and codes for one of the 11 type I acidic keratins.[27–29] The typical clinical features of monilethrix show wide variation within and between families. However, the clinical presentation

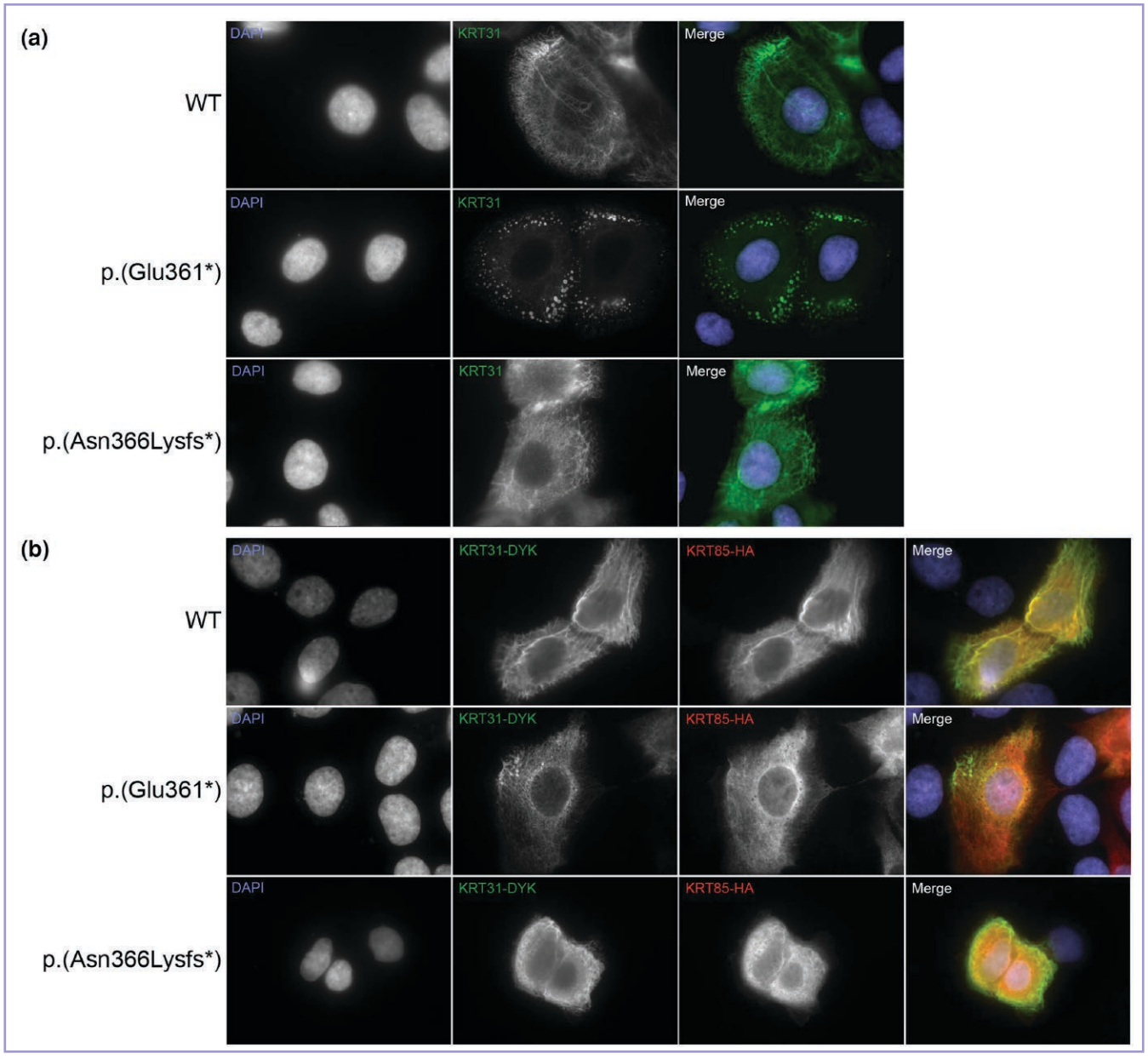

Figure 3 Immunofluorescence of keratins 31 and 85 in HaCaT cells transfected with a single- or double-expression system. (a) Localization of keratin 31 in HaCat cells. Wildtype (WT) keratin 31 formed filaments in the cytoplasm, compatible with cytoskeletal localization. p.(Glu361*) exhibited vesicular-like structures in the cytoskeleton that were formed by keratin 31 (green), whereas p.(Asn366Lysfs*) formed the same pattern as the WT. (b) Co-localization of keratin 31 (green) and keratin 85 (red). WT keratins 31 and 85 formed filaments in the cytoplasm, compatible with cytoskeletal localization. A co-expression system with p.(Glu361*) keratin 31 showed the vesicular-like structures in the cytoplasm. The structures included keratin 31 without keratin 85. The nonsense variant p.(Asn366Lysfs*) showed similar morphology to the WT. DAPI, 4′,6-diamidino-2-phenylindole.

provides no insight into the underlying genetic cause, as none of the known causal genes results in a distinct phenotype. This was also the case for the present *KRT31* variant c.1081G>T; p.(Glu361*). Notably, recent research in sheep has indicated a correlation between wool fibre diameter and variants in *KRT31*, consistent with the hair diameter changes observed in monilethrix.[30]

Type I keratins are present in several tissues, with their highest expression being found in epithelial cells, hair and nails.[31,32] Research has shown that keratin 31, which was previously termed hHa1, forms heterodimers with the basic keratin 85, encoded by *KRT85*.[26] A previous report in 2006 showed that biallelic pathogenic variants in *KRT85* cause pure hair and nail ectodermal dysplasia (MIM: 602032).[33] Interestingly, despite the absence of periodic changes in hair diameter, some of these patients show a hair phenotype that is similar to that observed in patients with monilethrix, with the preferential location being the back of the head.[34–36] Similarly, the irregularly shaped and fragile nails of the affected individuals in our study are similar to the nail phenotypes of individuals who were reported to carry pathogenic *KRT85* variants causative for pure hair and nail ectodermal dysplasia in a subsequent study from 2010.[36] Keratin 31 has previously been shown to be expressed in

the nail matrix and to be present throughout the entire upper layers,[32] and – indeed – co-expressed with keratin 85 in the lower layers where its expression is initiated.[37] Based on the similarities of the hair and nail phenotypes of individuals carrying pathogenic *KRT85* variants from previous reports and our patients with *KRT31* variants, it can be suggested that the keratin 31–keratin 85 heterodimers play an essential role in healthy hair and nail growth.

The *KRT31* pathogenic variant c.1081G>T; p.(Glu361*) leads to a stop mutation that abolishes both the final three amino acids of the 2B domain and the complete tail domain of keratin 31. Prediction tools for NMD and our RT-qPCR analysis did not generate evidence that the mutant transcript was subjected to NMD.[38–41] Previous research found that the splicing variant c.1097+1G>A leads to a similar truncated protein p.(Asn366Lysfs*).[24] However, in contrast to p.(Glu361*), this protein is characterized by the retention of the entire 2B domain. According to AlphaFold2 (https://alphafold.com/) prediction, this truncation leads to a deletion of the C-terminal two α-helical turns within the coiled coil assembly of two antiparallel helices (α_1 54–206; α_2 221–366) of the keratin 31 structure. Interestingly, the additionally deleted six amino acids (Glu_{361}–Ser–Glu–Asp–Cys–Asn_{366}) in the pathogenic variant c.1081G>T; p.(Glu361*) vs. the benign variant p.(Asn366Lysfs*) contain a surface-accessible cysteine residue (Cys365). As the corresponding acidic and basic keratin heterodimers align their termini with the termini of other heterodimers via the formation of disulfide bonds to form a protofilament of the IFs,[16] we hypothesize that deletion of Cys365 causes the severeness of the Glu361* variant vs. the Asn366Lys* variant. According to gnomAD, the frequency of the latter variant in the European population is 2.5%, and individuals with this variant show no abnormalities of the hair shaft.[24] This is consistent with the fact that this variant is functional and results in the construction of normal IFs.[24] A similar behaviour was demonstrated in our cell culture experiments with the binding partner keratin 85. Specifically, WT keratin 31 and the benign variant showed similar translation levels, and both showed a homogeneous cytoskeletal localization throughout the cytoplasm that overlapped with the localization signal from keratin 85. In contrast, the monilethrix-associated mutant protein showed a significantly lower abundance in the cells and an aberrant presentation in the form of vesicular-like structures, which accumulated near the cell membrane and did not co-localize with keratin 85. These observations confirm that, despite the similarity of the two mutant proteins, p.(Glu361*) has a pathological effect, which is probably related to the attenuation of its functionality or availability in the cell. We also hypothesize that the precise location of nonsense variants in *KRT31* – and thus whether or not the entire 2B domain of the protein is retained – has a strong effect on the pathogenicity of the resulting protein. Notably, keratin 31 mutants neither affect the abundance nor the cytolocalization of keratin 85. Therefore, we conclude that p.(Glu361*) is unlikely to exert a dominant negative effect by influencing keratin 85.

Interestingly, keratin aggregation and the formation of granules have also been described in other conditions arising secondary to pathogenic variants in keratin genes. In a knockout mouse model for keratin 2, the aggregation of its binding partner – keratin 10 – was observed, while deletion of *Krt10* led to an aberrant aggregation of keratin 2, hyperkeratosis and inflammation.[42] A previous study found that pathogenic variants in *KRT14*, which cause epidermolysis bullosa simplex (EBS), form similar abnormal structures. Keratin rings and solid aggregates were seen in the cytoplasm close to the cell edges in keratinocytes from affected individuals, which is similar to the present findings for the pathogenic p.(Glu361*) variant found in people with monilethrix.[43] In general, the keratin rings and aggregates were mostly detected after mechanical stress, suggesting that the mutated keratin 14 was unable to maintain the mechanical properties of the keratinocytes.[43] Desmosomes also localize to the cell membranes, as they are responsible for cell–cell adhesions. Desmosomal or hemidesmosomal IF-associated proteins were retained in the keratin rings formed by mutant keratin 14 associated with EBS, which suggests that desmosomes may be implicated in EBS pathogenesis. Given that monilethrix is also associated with biallelic pathogenic variants in *DSG4*, which encode for a desmosomal cadherin, the aberrant vesicular-like structures formed by mutant keratin 31 may also affect the desmosome.

A further intriguing possibility is that pathogenic variants in other monilethrix-associated keratins may lead to the formation of similar vesicular-like structures. Accordingly, further studies of *KRT81*, *KRT83* and *KRT86* variants are warranted to clarify whether the autosomal dominant form of monilethrix is always related to the presence of such aberrant structures.

All of the affected individuals reported herein were of German ancestry. Haplotype analysis revealed that they all shared a common haplotype, which has an allele frequency of around 17% in the European population. Therefore, geographical and genetic data suggest that the *KRT31* variant c.1081G>T; p.(Glu361*) may have derived from a single mutational event. Further research is required to determine whether 1081G>T; p.(Glu361*)-related monilethrix exists in other populations and whether additional pathogenic variants in *KRT31* are present.

In summary, we have identified a pathogenic variant in *KRT31* as the molecular cause of autosomal dominant monilethrix in nine individuals from four unrelated families. Cell culture experiments in cultured keratinocytes demonstrated that this pathogenic variant led to the production of a mutant keratin 31, which formed vesicular-like structures, and that these accumulate around the cell membrane of keratinocytes and show impaired co-localization with the binding partner keratin 85.

The discovery of a new genetic cause for autosomal dominant monilethrix is expected to increase the molecular diagnostic yield for hair and nail phenotypes and improve genetic counselling for individuals and families with monilethrix.

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Conflicts of interest

The authors declare no conflicts of interest.

Data availability

The data underlying this article are available from the corresponding author upon reasonable request.

Ethics statement

The study was performed in accordance with the principles of the Declaration of Helsinki. Ethical approval was obtained from the ethics committee of the Medical Faculty of the University of Bonn, Germany (007/10-A).

Patient consent

All participants or legal guardians provided written informed consent to study participation prior to blood/saliva sampling. Written informed consent for the publication of all clinical photographs was obtained from the affected individuals or their legal guardians.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website.

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3.2 Publication 2:

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CED
Clinical and Experimental Dermatology
Genetic Report

A *TMC8* splice variant causes epidermodysplasia verruciformis in a Pakistani family

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Epidermodysplasia verruciformis (EV) is a rare autosomal recessive skin disease that is characterized by persistent disseminated flat warts and pityriasis versicolor-like lesions associated with abnormal susceptibility to the human beta papillomavirus.[1] The risk for carcinoma is quite high, especially in sun-exposed areas. More than half of patients with typical EV have pathogenic variants in either *TMC6* or *TMC8*.[2,3] Biallelic pathogenic variants in *CIB1* have also been associated with EV.[4]

Here, we studied the genetic basis of EV in a consanguineous family from Pakistan (Figure 1a) using a molecular diagnostic approach. Four of 11 siblings developed the phenotype in early childhood. According to self-report, preliminary manifestations include vomiting, diarrhoea and warts-like lesions, which initiated on the forehead, and extended towards the neck, hands, arms and feet and got intensified upon sun exposure (Figure 1b–e).

Blood samples were collected from the parents and five siblings, three of whom are affected. DNA was extracted from peripheral blood leucocytes according to standard methods. All participants provided written informed consent, in accordance with the Declaration of Helsinki principles.

We performed whole exome sequencing in one of the affected siblings (IV:5) at the Cologne Center for Genomics (Cologne, Germany). Data analysis were performed using the Varbank pipeline v.2.0 (https://varbank.ccg.uni-koeln.de/). We identified the homozygous single-base exchange c.668+5G>A in intron 6 of *TMC8*. The variant and its co-segregation with the phenotype was confirmed by Sanger sequencing (Figure S1; see Supporting Information).

In silico analysis via the HSF Pro tool (Genomnis, Marseille, France) suggested that the variant c.668+5G>A leads to the impairment of the 5′ donor splice site of intron 6. Owing to the lack of RNA samples from the patients, we used an *in vitro* approach to analyse the effects of this variant on splicing. We used the Exon trap vector (MoBiTec, Göttingen, Germany), to drive the transcription of the region spanning exons 5–7 of *TMC8* in HEK293T cells, either in the absence (wildtype plasmid; WT) or presence of the identified variant (mutated plasmid).

We observed that the expression of the WT plasmid led to the production of four transcripts: A+, A, B+ and B. A is identified as the canonical transcript, containing all three exons, A+ is the same as A but contains an additional 40-bp long intronic fragment, B contains only exon 7 and B+ is the same as B but contains the same intronic fragment found in A+ (Figure 2, Figure S2; see Supporting Information). In addition, the mutated plasmid expresses two additional transcripts called M and M+ (exon 5 plus exon 7 with or without the 40-bp long intronic fragment) (Figure 2). These results suggest that the variant c.668+5G>A disturbs the canonical splicing, leads to overexpression of some transcripts and leads to the generation of a new transcript in which exon 6 is skipped (detailed description in Appendix S1; see Supporting Information).[5]

In the affected family members, the identified mutation affects the G nucleotide that is 3-bp adjacent to the conserved 5′ GT of the donor splice site and thus leads to disruption of the GTRAG motif,[6] which most 5′ splice sites start with. Therefore, it is reasonable to expect that it has an

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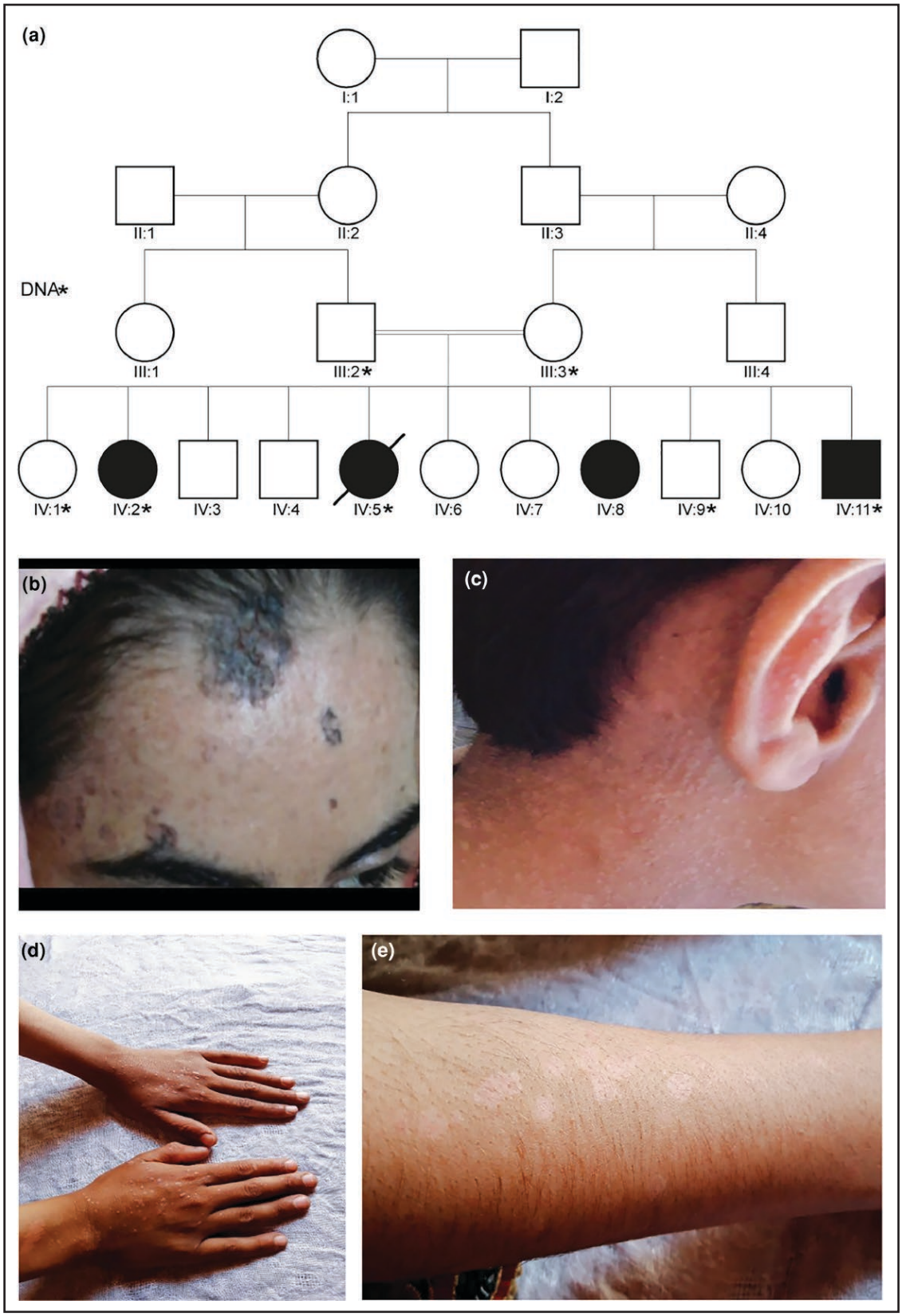

Figure 1 Pedigree, clinical features of patients, sequencing results and gel electrophoresis. (a) Pedigree of the family studied. The affected siblings are depicted with black circles and squares. Double lines mark consanguinity. *Availability of DNA from the respective individual. (b) Individual IV:5 presented with multiple asymptomatic violaceous papules and plaques from childhood, hyperpigmented plaques on the face with ill-defined margins with some scales on the forehead. She exhibited eruption of lesions with pus or fluid when exposed to sunlight suggestive of skin keratosis and later had cancer and died at the age of 24 years. (c) Dominant verrucous papules and hypopigmented macules present on the neck of individual IV:11. (d,e) Photographs show lesions on the hands and arm of individuals IV:2 and IV:5, respectively, that can cause itching and redness when exposed to sun.

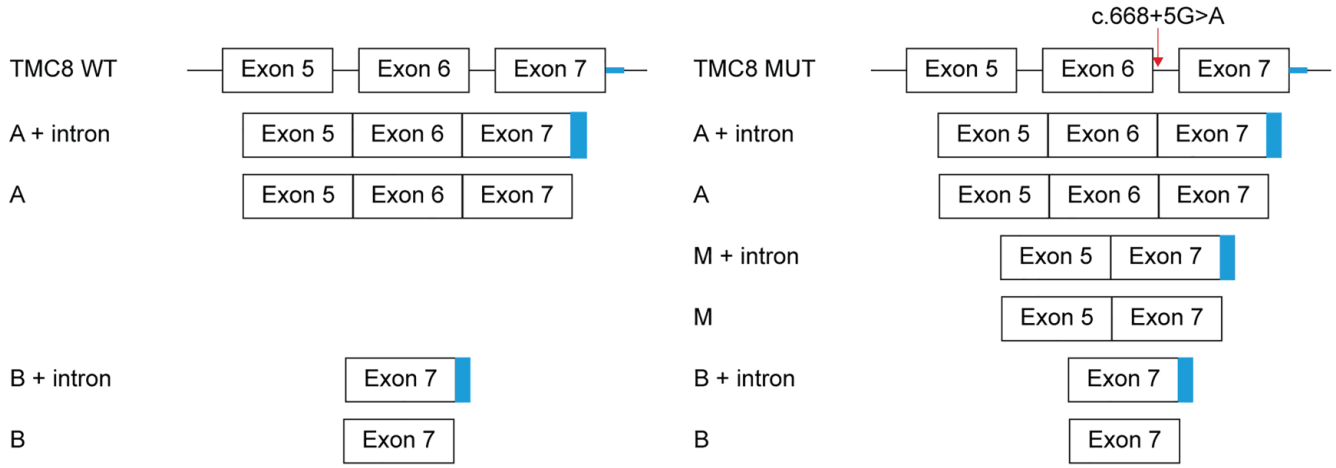

Figure 2 Diagram depicting the *TMC8* splice transcripts generated from expression of the wildtype (*TMC8* WT) vs. mutated Exon Trap vector (*TMC8* MUT). MUT expressed two more transcripts compared with the WT corresponding to an exon 6 skipping event with (M+) or without (M) retention of an intronic sequence that is depicted by blue.

effect on splicing. Owing to the lack of RNA samples from this family, we could, however, examine this effect only *in vitro* and observed skipping of exon 6 to be a consequence of the identified mutation. Accordingly, we cannot exclude that there is a different splicing outcome in the skin of the affected family members.

In conclusion, we have expanded the mutation spectrum of EV by identification of a homozygous disease-causing variant in *TMC8* in a Pakistani family. The phenotype of EV in the affected individuals is most probably caused by an aberrant splicing process.

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Conflicts of interest

The authors declare they have no conflicts of interest.

Data availability

Data are available on request from the corresponding author.

Ethics statement

Ethical approval: granted by the ethics committee of the Institute of Biochemistry, University of Balochistan, Quetta, Pakistan. Informed consent: all patients gave written, informed consent for participation and publication of their case details and images.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website.

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CPD questions

Learning objective

To demonstrate knowledge of the underlying genetic defect and clinical manifestations of epidermodysplasia verruciformis.

Question 1

Which genes are involved in epidermodysplasia verruciformis (EV)?

(a) *TMC1*.
(b) *KRT5*.

(c) *TMC8*.
(d) *KRT85*.
(e) *COL7A1*.

Question 2

Which of the following features occur in epidermodysplasia verruciformis (EV)?

(a) Persistent disseminated flat warts and pityriasis versicolor-like lesions.
(b) Sun-exposed areas have lower susceptibility to cutaneous malignancies.
(c) Abnormal susceptibility to herpes zoster.
(d) The patient's lesions heal over time.
(e) High susceptibility to the development of melanoma skin cancers.

3.3 Publication 3:

Nanda A, **Xiong X**, AlLafi A, Cesarato N, Betz RC. Cole disease due to a novel pathogenic variant in the *ENPP1* gene. J Eur Acad Dermatol Venereol. 2022; 36(7): e559-e561 https://doi.org/10.1111/jdv.18028

LETTER TO THE EDITOR

Cole disease due to a novel pathogenic variant in the *ENPP1* gene

Editor,

Cole disease (COLED, MIM #615522) is a rare genodermatosis characterized by dyspigmentation and punctate palmoplantar keratoderma (PPPK) of which only 13 families have been reported in the literature to date.[1–8] We report an Indian family with Cole disease with a novel pathogenic variant in *ENPP1* in the somatomedin-B-like2 (SMB2) domain.

A 4-year-old girl presented with slowly progressive hypopigmented lesions on upper and lower extremities (Fig. 1a,b) and trunk since birth. Subsequently, she developed few hyperpigmented lesions. She was also noticed to have painless punctate keratoderma of the palms and soles (Fig. 1c,d) since early infancy. Family history revealed hypopigmented lesions in her father (Fig. 1e), paternal aunt, grandmother and great-grandfather (Fig. 2a). Her father did not have PPPK, and he was unaware of PPPK in other family members. The routine laboratory work-up and abdominal ultrasonography both in the proband and her father were reported normal.

After Bio Chika (Edited).docx written informed consent was signed, in accordance with the principles of the declaration of Helsinki, direct Sanger sequencing of *ENPP1* exons 4 and 5, coding for SMB2 domain of the protein, was performed in the proband and her father. The results revealed the missense variant c.490T>C; p. Cys164Arg (Fig. 2b). The variant is predicted to be pathogenic by MutationTaster (http://mutationtaster.org/), and has a combined annotation-dependent depletion (CADD) score of 27.8, which indicates that the variant is among the top 1% of the deleterious variants in the human genome, and was not described in the database gnomAD (https://gnomad.broadinstitute.org/).

Classical features of Cole disease include cutaneous dyspigmentation, commonly hypopigmented macules of varying sizes and rarely hyperpigmented macules predominantly located on the extremities, PPPK and infrequently ectopic calcifications. It is caused by pathogenic variants in *ENPP1* that encodes ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1); and both autosomal dominant (AD) and autosomal recessive (AR) modes of inheritance have been described.[3,5,6] Hypopigmented macules start from birth to 18 months. The ultrastructural studies of hypopigmented macules have shown the presence of disproportionately large numbers of melanosomes in the cytoplasm and dendrites of melanocytes but the reduced number of

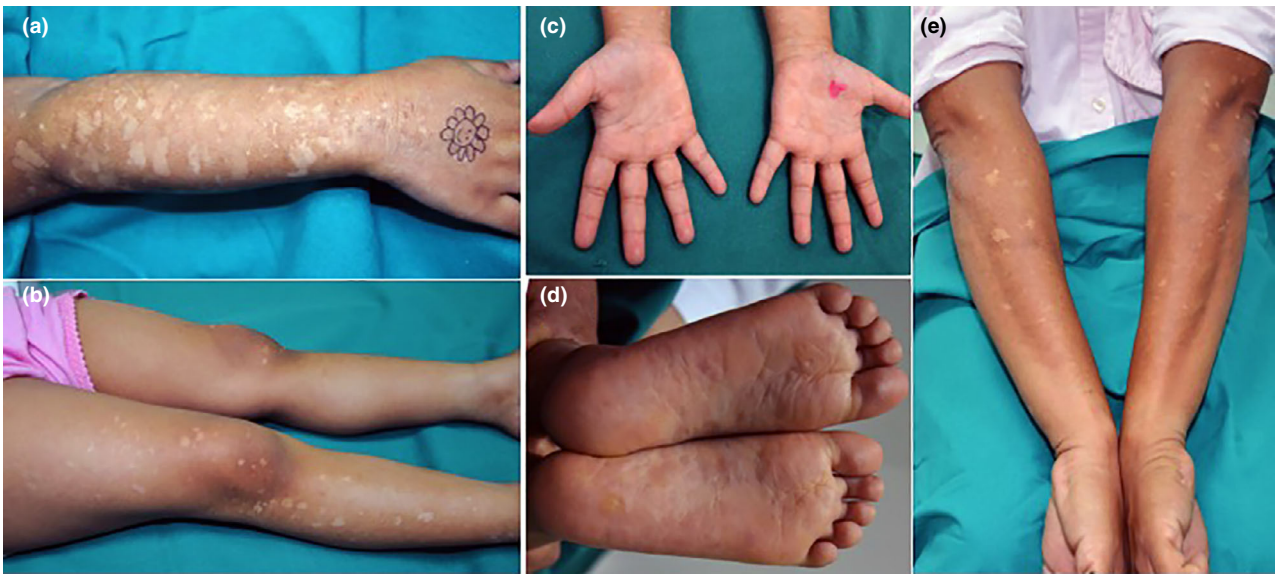

Figure 1 Proband with (a) Hypopigmented macules on the forearm and dorsum of the hand with few scattered hyperpigmented macules; (b) hypopigmented macules on the lower extremities; (c, d) punctate keratoderma on the palms and soles; and (e) hypopigmented lesions on the forearms of the father.

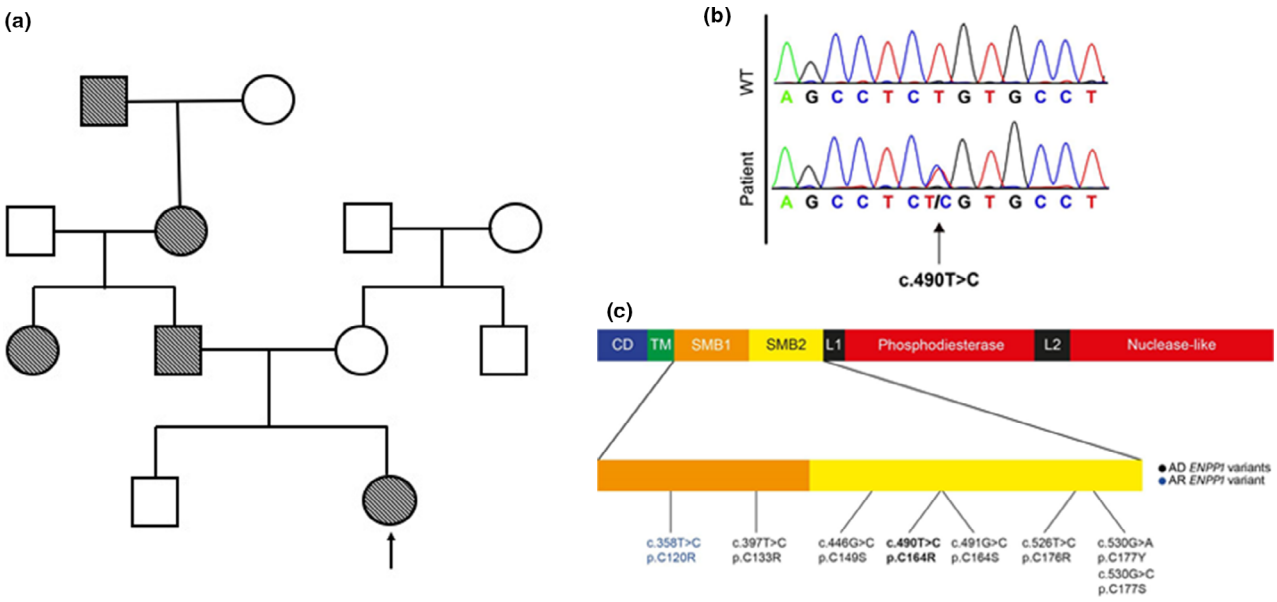

Figure 2 (a) Family pedigree with five affected members (with back pattern fill); (b) electropherograms of the index patient compared with the respective wild-type (WT) sequence; and (c) ENPP1 domains, all the known variants in the SMB1 and SMB2 domains, associated with autosomal dominant (AD) and autosomal recessive (AR) Cole disease, are shown; the newly identified variant is depicted in bold.

melanin content in the keratinocytes suggest an impaired melanosome transfer.[2]

The molecular basis of COLED was revealed recently by the identification of pathogenic variants in *ENPP1*.[3] ENPP1 is involved in the regulation of calcification and mineralization, blood sugar control and maintenance of epidermal integrity.[3] It has also been shown to inhibit insulin signalling through the interaction between SMB2 domain and insulin receptors. Insulin signalling plays a critical role in epidermal homeostasis including keratinocyte differentiation, and melanosome uptake by keratinocytes.[3] Phenotypic characteristics of Cole disease have been suggested to be related to the impaired inhibition of insulin signalling pathway by ENPP1.[3] Novel heterozygous variants were subsequently reported in the SMB1 and SMB2 domains of *ENPP1* in two unrelated families, thus, supporting the role of both these domains in the pathogenesis of COLED.[5] Two members in one of these families had no skin lesions supporting incomplete penetrance. Chourabi *et al.*[6] reported three families from Tunisia with homozygous variants in the SMB1 domain and proposed that the patients with homozygous variants have a severe phenotype with a widespread dyspigmentation including hypo- and hyperpigmented macules on the trunk and extremities. Ectopic calcifications are rarely encountered in both AD and AR variants of COLED.[3,5,6] Figure 2c shows all pathogenic variants reported to date for COLED. Our patients showed a novel variant in *ENPP1* in the SMB2 domain with phenotypic variability among the two patients genetically tested. The proband had widespread dyspigmentation (both hypo- and hyperpigmented macules), and PPPK, but her father had only hypopigmented macules localized to the extremities. We propose that generalization of the skin lesions and the presence of hyperpigmented macules are not exclusive to the AR variant. Of interest, another pathogenic variant affecting the same codon 164 (p. Cys164Ser) has already been associated with COLED, underlining the important role of this amino acid.[3] With a novel pathogenic variant in the SMB2 domain of *ENPP1*, we add onto the mutation spectrum of the COLED.

Acknowledgements

The patients in this manuscript have given written informed consent to publication of their case details.

Conflict of interest

None.

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Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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4. Discussion with references

4.1 The importance of research on skin and hair disorders

Several large studies on the burden of skin diseases have been performed, showing that skin diseases may severely impair social and personal daily life, and have psychological consequences, such as depression and anxiety (Dalgard et al., 2015). The research including patients across Europe indicated that skin diseases may cause feelings of stigmatization (Gisondi et al., 2023). Consequently, research into skin and hair disorders is imperative for public health and prevention. To date, investigating the genetics of monogenic skin and/or hair disorders is still the classical and essential method to unravel the relationship between genes and the normal or aberrant development of skin and its appendages, such as hair follicle.

There are many positive outcomes from research into skin and hair disorders. By studying new variants and genes, molecular diagnostics for rare and inherited disorders can be improved accordingly. In addition, such research enhances our knowledge of ectodermal biology and tissue regeneration, offering broader scientific insights. Our studies serve as the foundation for clinical research, with all outcomes contributing to advancements in translational medicine.

4.2 Mutations identified for EV and COLED, respectively, in *TMC8* and *ENPP1*

In the *TMC8* paper, we describe the splice variant c.668+5G>A in four affected individuals from a consanguineous family presenting with epidermodysplasia verruciformis (Xiong et al., 2023). We observed that different transcripts could be expressed, in particular, exon 6 has been skipped from the cell experiment that we made. This indicated the variant disrupts the GTRAG motif at the donor splice site, leading to aberrant splicing and the generation of non-canonical transcripts (Abramowicz and Gos, 2018; Sibley et al., 2016). Although the splicing effect has been confirmed *in vitro*, we cannot rule out alternative splicing in the skin of affected family members. This study broadens the mutational spectrum and highlights the role of aberrant splicing in the pathogenesis of EV.

In the *ENPP1* study, we identified the novel pathogenic variant c.490T>C; p. Cys164Arg as the underlying genetic cause of COLED and analysed the associated phenotypic variability within an affected family (Nanda et al., 2022). Previous studies indicated that the

phenotype of COLED varies from no skin lesions to severe conditions, due to heterozygous or homozygous variants (Chourabi et al., 2018; Schlipf et al., 2016). In our study, although the affected individuals carried the same heterozygous variant, the proband manifested with widespread dyspigmentation and PPPK, whereas the father only presented with hypopigmented macules on the extremities. This suggests that the generalization of skin lesions and hyperpigmented macules is not limited to AR variants. Notably, the amino acid (AA) Cysteine at codon 164 was abnormally replaced by Serine in a previously reported study, while in our study, it was replaced by Arginine (Eytan et al., 2013). Therefore, it highlights the critical importance of this AA.

4.3 *KRT31* as a novel gene for monilethrix

Our study is the first to present the identification and characterization of *KRT31* as a new gene for autosomal dominant monilethrix. *KRT31* encodes for keratin 31, which belongs to the type I acid hair keratin family (Xiong et al., 2024). Nine affected individuals from four unrelated families carried the nonsense variant c.1081G>T; p.(Glu361*) in *KRT31*. The type II basic keratin genes (including three keratin genes previously described for monilethrix) are located on chromosome 12, whereas the type I acid keratin genes (including *KRT31*) are clustered on chromosome 17 (Rogers et al., 1995; Rosenberg et al., 1988; Yoon et al., 1994).

In clinical diagnosis, typical manifestations of monilethrix vary in and between families. However, also the genetic cause of monilethrix cannot be recognized through the clinical symptoms, as no distinct phenotype can be observed in individuals with variants in different genes causing monilethrix. This observation was also made in the case of the individuals with *KRT31* variant from our study. Interestingly, a study on sheep wool traits have demonstrated a correlation between *KRT31* mutation and wool fiber diameter, aligning with the changes in hair diameter observed in monilethrix (Sulayman et al., 2018).

Previous research described that the basic keratin 85 forms heterodimers with keratin 31, and pathogenic variants in *KRT85* were initially identified as causal of pure hair and nail ectodermal dysplasia (MIM: 602032) (Langbein et al., 2007; Naeem et al., 2006). Notably, although the observation of affected hair with periodic diameter changes was absent, some of the patients exhibited a hair phenotype similar to monilethrix, with the preferential affected location being the occiput, and abnormal nails (fragile or irregular shaped nails)

similar to the nail phenotype of the patients presented in our study (Amico et al., 2019; Shimomura et al., 2010; Zhu and Zeng, 2023). Considering the co-expression of keratin 31 and keratin 85 in certain nail layers (De Berker et al., 2000; Perrin et al., 2004), along with the similarities in hair and nail phenotypes between the two disorders, we could suggest that the growth of hair and nail growth are indeed connected with keratin 31-keratin 85 heterodimers.

The pathogenic variant p.(Asn366Lysfs*) leads to a stop mutation which cause deletion of both the last three amino acids in the 2B domain and the whole tail structure. In particular, the loss includes Cys365 (surface-accessible cysteine residue), which is crucial for disulfide bond formation in IF protofilaments (Steinert et al., 1985). Consequently, we proved that the premature stop codon did not lead to nonsense-mediated mRNA decay by using RT-qPCR analysis (Lappalainen et al., 2013; Lindeboom et al., 2016; Nagy and Maquat, 1998; Rivas et al., 2015). Previous research reported that variant c.1097+1G>A leads to a similar truncated protein p.(Asn366Lysfs*) (Winter et al., 1997). In comparison with our pathogenic variant, the complete 2B domain is retained in this protein. According to AlphaFold2 (https://alphafold.com/) prediction, the c.1081G>T; p.(Glu361*) variant leads to the loss of two α-helical turns on the C-terminus of the keratin 31 structure. Overall, we hypothesize that the absence of Cys365 causes the severeness of the p.(Glu361*) variant rather than the p.(Asn366Lysfs*) variant. According to gnomAD, the latter variant occurs at a frequency of 2.5% in the European population and carriers are not reported to have any hair shaft abnormalities. This leads to the assumption that this variant is functional and constructs normal IFs (Winter et al., 1997). Our cell culture experiments demonstrated that wildtype keratin 31 and this benign variant also behave similarly. In contrast, the p.(Glu361*) variant causes lower abundance of the protein and localization abnormalities in cell culture experiments, leading to vesicular-like structures that do not colocalize with keratin 85. This result suggests that the pathogenicity of p.(Glu361*) stems from reduced protein functionality or availability, rather than a dominant-negative effect on keratin 85. Therefore, it further highlights the importance of retaining the 2B domain for proper keratin 31 function.

During our study, we noticed that keratin aggregation and the formation of granules were also described in both human and mouse models, in particular, abnormal keratin could not maintain the cell integrity, and desmosomal IF-associated proteins were included in

the aggregation (Fischer et al., 2014; Russell et al., 2004). Given that *DSG4*, a desmosomal cadherin gene, is implicated in monilethrix, it is plausible that mutant keratin 31 also impacts the desmosome. This raises the possibility that other monilethrix-associated keratin genes (*KRT81*, *KRT83*, and *KRT86*) may lead to similar vesicular-like structures. Further investigation into these keratins is warranted to determine whether such aberrant structures consistently underlie the pathogenesis of monilethrix.

Interestingly, all affected individuals in this study were of German descent and shared a common haplotype with a 17% allele frequency in Europeans, suggesting that the variant c.1081G>T; p.(Glu361*) originated from a single mutational event. Further research is needed to explore the presence of this variant in other populations and of additional *KRT31* variants causative for monilethrix.

In summary, we identified a pathogenic *KRT31* variant as cause of autosomal dominant monilethrix, showing that it produces a truncated form of keratin 31 forming vesicular-like structures with impaired co-localization to keratin 85. These results enhance molecular diagnostics and genetic counselling for affected individuals and families.

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6. Statement on own contribution

The work presented in the present thesis was carried out at the Institute of Human Genetics / University Hospital Bonn under the supervision of Prof. Dr. med. Regina C. Betz.
The specific contributions to the publications included in this thesis are detailed below:

1. A *TMC8* splice variant causes epidermodysplasia verruciformis in a Pakistani family
In this study, where I am the lead author and have written the first version of the manuscript myself, the academic work was planned by Prof. Betz and Prof. Ayub.
Data collection: The Sanger sequencing and cell experiments were carried out by me independently. Samples of affected individuals were collected by S.A. Uddin and other researchers from Pakistan.
Evaluation: Exome sequencing and immunoblotting data were analysed by me independently.
Interpretation: Electropherograms and clinical data were interpreted by me independently. S. Munir interpreted the specific clinical features.

2. Cole disease due to a novel pathogenic variant in the *ENPP1* gene
In this study, where I am a co-author, the academic work was supervised and planned by Dr. Nanda and Prof. Betz.
Data collection: I performed the Sanger sequencing of the affected individuals independently. Dr. Nanda and Prof. Allafi were responsible for sample collection.
Evaluation: I conducted the analysis of the exome sequencing data independently.
Interpretation: Electropherograms were interpreted by me independently. Dr. Nanda interpreted the clinical features.

3. A nonsense variant in *KRT31* is associated with autosomal dominant monilethrix
In this study, I am the lead author and have written the first version of the manuscript myself. The work was designed by me and was supervised by Prof. Betz and Dr. Basmanav.

Data collection: I performed the Sanger sequencing and cell experiments independently. Samples of affected individuals were collected by Prof. Betz, Prof. Hamm, Dr. Demuth, and Dr. Oji.
Evaluation: The data from exome sequencing and cell experiments were analysed by me independently. The haplotype analysis was performed by me. The protein modelling was performed by Prof. Geyer.
Interpretation: The figures and clinical data were interpreted by me. The protein modelling data were interpreted by Prof. Geyer.

I confirm that I have written this thesis independently and have not used any sources or aids other than those specified by me.

7. Curriculum Vitae

Additional publications

Li Y, **Xiong X**, Cesarato N, Wehner M, Basmanav FB, Betz RC: First East Asian case of uncombable hair syndrome. **J Dtsch Dermatol Ges** 22;1433-1435, 2024

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