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ORIGINAL ARTICLE

De novo mutation in *KITLG* gene causes a variant of Familial Progressive Hyper- and Hypo-pigmentation (FPHH)

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Abstract

Familial Progressive Hyper- and Hypopigmentation is a pigmentary disorder characterized by a mix of hypo- and hyperpigmented lesions, café-au-lait spots and hypopigmented ash-leaf macules. The disorder was previously linked to *KITLG* and various mutations have been reported to segregate in different families. Furthermore, association between *KITLG* mutations and malignancies was also suggested. Exome and SANGER sequencing were performed for identification of *KITLG* mutations. Functional in silico analyses were additionally performed to assess the findings. We identified a de novo mutation in exon 4 of *KITLG* gene causing NM_000899.4:c.[329A>T] (chr12:88912508A>T) leading to NP_000890.1:p.(Asp110Val) substitution in the 3rd alpha helix. It was predicted as pathogenic, located in a conserved region and causing an increase in hydrophobicity in the KITLG protein. Our findings clearly confirm an additional hot spot of KITLG mutations in the 3rd alpha helix, which very likely increases the risk of malignancies. To our knowledge the present study provides the strongest evidence of association of the KITLG mutation with both Familial Progressive Hyper- and Hypopigmentation and malignancy due to its' location on somatic cancer mutation locus. Additionally we also address difficulties with classification of the unique phenotype and propose a subtype within broader diagnosis.

KEYWORDS

exome sequencing, familial progressive hyper- and hypo-pigmentation, gain-of-function, KITLG

Mario Gorenjak and Nino Fijačko contributed equally to this work.

1 | INTRODUCTION

Inherited autosomal dominant Familial Progressive Hyper- and Hypopigmentation (FPHH) is a pigmentary disorder characterized by a mix of hypo- and hyperpigmented lesions, café-au-lait spots (CALS) and hypopigmented ash-leaf macules (Amyere et al., 2011). *KITLG* gene (MIM: 184745) encoding KIT ligand protein has been previously reported as a disease-causing gene for FPHH. Additionally, autosomal dominant Familial Progressive Hyperpigmentation (FPH) and autosomal recessive Dyschromatosis Universalis Hereditaria 2 (DUH2, MIM: 612715) were also linked to the *KITLG* locus in chromosome 12 (Bukhari, El-Harith, & Stuhrmann, 2006; Stuhrmann et al., 2008; Wang et al., 2009). Recently, FPHH and FPH have been joined under new diagnosis Familial Progressive Hyper- with or without Hypopigmentation (FPHH, MIM: 145250). KITLG binds its receptor c-Kit, triggering the RAS/MAPK signaling pathway, which plays a critical role in the regulation of cutaneous pigmentation (Picardo & Cardinali, 2011). To this date, various FPHH-causing substitutions in KITLG within or adjacent to conserved VTNN motif have been reported in families from China, United States, Denmark and United Kingdom (NP_000890.1:p.(Val33Ala), NP_000890.1:p.(Thr34Pro), NP_000890.1:p.(Thr34Asn), NP_000890.1:p.(Thr34Ile), NP_000890.1:p.(Asn35Ile), NP_000890.1:p.(Asn36Ser), NP_000890.1:p.(Val37Gly)) (Amyere et al., 2011; Cuell et al., 2015; Wang et al., 2009; Zhang et al., 2016). In addition, a novel substitution NP_000890.1:p.(Glu113Lys) located within another ligand-receptor interaction site has been lately reported (Kato et al., 2020). It was also reported that *KITLG* polymorphisms are associated with different malignancies (Hang et al., 2017; Kanetsky et al., 2009). Melanomas, pharyngeal and papillary thyroid cancers were observed in individuals with NP_000890.1:p.(Thr34Asn) and NP_000890.1:p.(Val37Gly) substitutions (Cuell et al., 2015). However, pathogenic somatic mutations associated with cancer are located within KITLG alpha helix, whereas FPHH causing substitutions are located within VTNN motif outside of the somatic mutation region (Cuell et al., 2015). Thus, question of association between FPHH and malignancy should be further addressed in terms of coincidence.

Herein we report pathogenic *de novo KITLG* gene mutation NM_000899.4:c.[329A>T] leading to NP_000890.1:p.(Asp110Val) substitution located within another ligand-receptor interaction site in a Slovenian (Caucasian - Central Europe) pedigree with a history of unique combination of pigmentary abnormalities (previously not described in patients with *KITLG* mutations) and with colorectal cancer co-morbidity.

2 | MATERIALS AND METHODS

2.1 | Clinical information

We studied a Slovenian family of 3 affected individuals presenting the features of extensive systemic FPHH and 7 unaffected relatives (Figure 1). The study was conducted according to Helsinki declaration for human studies research. Written consent was obtained from all subjects and the study was approved by a local institutional review board (UKC-MB-KME-60/20).

The study probands GL2, GL3 and GL4 were referred to Department of Dermatology and Venereal Diseases, University Clinical Centre Maribor due to suspected Leopard syndrome. Affected individuals presented extensive hyperpigmented macules of different sizes with lentigo and CALS, and smaller number of hypopigmented macules with ash-leaf spots and also CALS, found in a widespread distribution, including the palms, soles, and lips (Figure 2). Hypopigmentation within hyperpigmented lesions (eg. CALS) and hyperpigmentation within hypopigmented lesions was also observed. Additionally, profound periorbital hyperpigmentation was noticed. The lesions began to develop during infancy and were

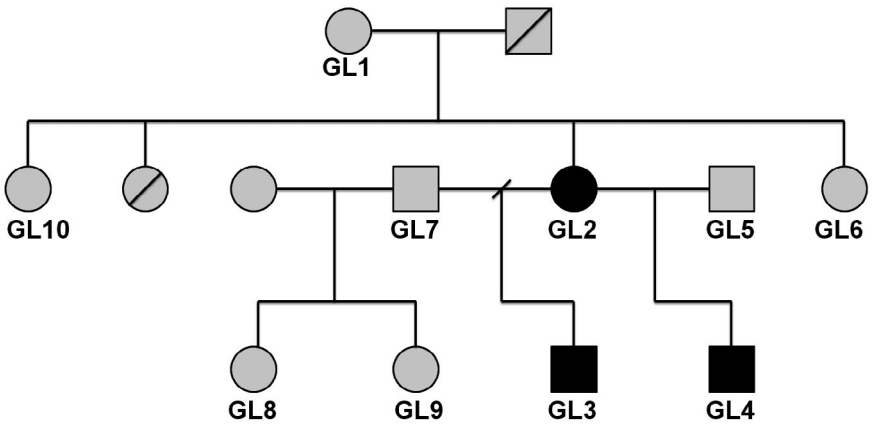

FIGURE 1 Segregation of familial progressive hyper- and hypopigmentation in the investigated Slovenian family. Black symbols indicate familial progressive hyper- and hypopigmentation

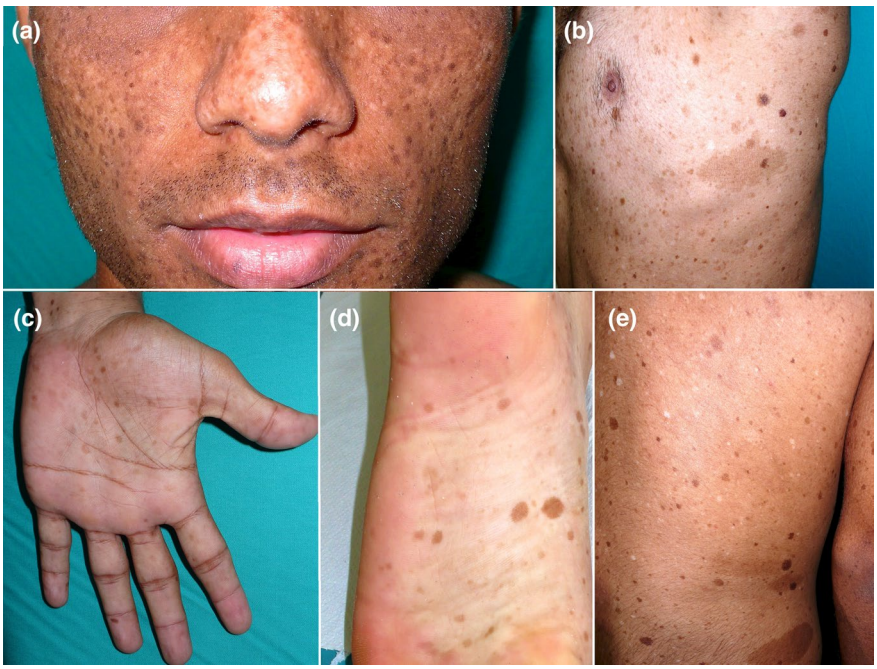

FIGURE 2 Clinical features of the proband GL3. A: face. B: left front upper body. C: palm. D: sole. E: right back upper body

increasing in size, number, and confluence with age, but stopped developing after puberty. Additionally, the skin was diffusely hyperpigmented, especially on photo-exposed skin. Interestingly, hypopigmentation was also more profound on these areas. By Fitzpatrick scale the probands GL3 and GL4 were categorized into type IV and proband GL2 into type V, but unaffected individuals were classified as type II (Fitzpatrick, 1988).

All three individuals were negative for mutations in *PTPN11*, *RAF1* and *BRAF*, thus Leopard syn. diagnosis was rejected. Skin specimens (hyperpigmented macule of the right knee, hypopigmented macule on the left leg and CALS on the right back) of 35-year-old proband GL3 were histologically examined by hematoxilyn-eosin (HE), SOX10, HMB45 and Masson-Fontana (MF) staining. Hyperpigmented macule has shown increased number of basal melanocytes, hyperpigmented basal keratinocytes and numerous melanophages in the upper dermis due to pigment incontinence (Figure 3A1). SOX10 and HMB45 staining showed increased number of basal melanocytes (Figure 3A2,A3) and MF staining showed numerous melanophages in the upper dermis due to pigment incontinence (Figure 3A4). Hypopigmented macule showed few melanophages in the upper dermis (Figure 3B1) with normal number of basal melanocytes (Figure 3B2,B3) and few melanophages in the upper dermis (Figure 3B4). CALS showed slightly increased number of basal melanocytes, hyperpigmented basal keratinocytes and melanophages in the upper dermis due to pigment incontinence (Figure 3C1) and slightly increased number of basal melanocytes (Figure 3C2,C3). MF showed an increased number of melanophages in the upper dermis due to pigment incontinence (Figure 3C4). The proband GL2 was also diagnosed with colorectal adenocarcinoma tubulare at age of 37.

2.2 | Mutation screening

Venous blood samples were collected into K_2EDTA tubes. DNA was extracted from peripheral blood mononuclear cells (PBMC) using TRI-reagent® (Merck, Darmstadt, Germany) according to manufacturer's instructions. Quality and integrity of extracted DNA were further checked using Synergy™ 2 spectrophotometer (Biotek, Winooski, VT, USA), using agarose gel electrophoresis and 2100 Bioanalyzer Instrument (Agilent, Santa Clara, CA, USA). Affected individuals GL2, GL3 and GL4 were screened for the mutation using next-generation sequencing (NGS) exome sequencing. Paired-end 2 × 150 bp exome sequencing was performed using Reagent Kit V2 (Illumina, San Diego, California), TruSight One Sequencing Kit (Illumina) and MiSeq system (Illumina). Bioinformatic analysis was carried out using an in-house built *MGPS* v4.1 pipeline for exome sequencing following best-practices (Van der Auwera et al., 2013). Obtained raw.*fastq* files were first assessed for quality control using *FastQC* software (Andrews, 2010). Technical sequences and sequencing adapters were trimmed using *Trimmomatic* 0.39 (Bolger, Lohse, & Usadel, 2014). Obtained reads were aligned to GRCh37/hg19 human reference genome using Burrows-Wheeler transformation *BWA* v0.7.12-r1039 and the *BWA-MEM* algorithm (Li & Durbin, 2010). Conversion to.*bam*, PCR duplicates marking and mate information fixation was carried out using *PicardTools* v2.20.1 (broadinstitute.github.io/picard/).

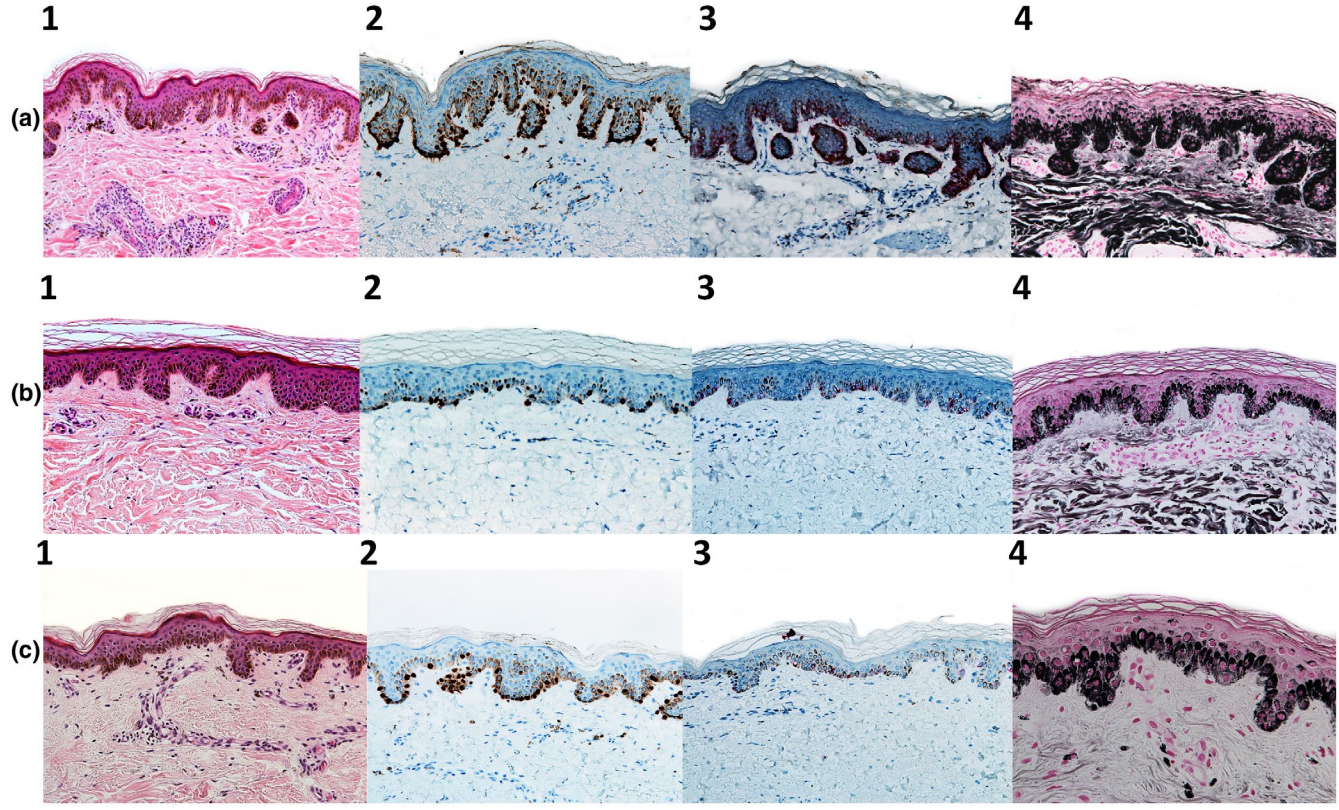

FIGURE 3 Histological examination of proband GL3. A: hyperpigmented macule of the right knee. B: hypopigmented macule on the left leg. C: Café-au-lait macule on the right back. 1: HE stain; 2: SOX10; 3: HMB45; 4: MF

Subsequently, base quality scores were recalibrated using *Genome Analysis Toolkit* (*GATK*) v3.8 (DePristo et al., 2011; McKenna et al., 2010). Variant calling was performed using *HaplotypeCaller* algorithm running in genomic VCF mode (Poplin et al., 2018). Joint calling was performed with other exome samples to increase robustness of the analysis using. Additionally, variant quality score recalibration, normalization and left-trimming were performed. Obtained.vcf files were annotated using *ANNOVAR* v2019-10–24 (K. Wang, Li, & Hakonarson, 2010).

Identified variant was further confirmed using SANGER sequencing in all included family members. Specific primers were designed using IDT OligoAnalyzer Tool (www.idtdna.com/pages/tools/oligoanalyzer). Primers were synthesized by MERCK (MERCK, Darmstadt, Germany). Primers were targeting exon 4 of *KITLG* gene: FW: 5′ CAGGCACTTGTAATCTGAGC 3′ and RV: 5′ ATAAAGGTGCCTCATTTTCC 3′. For SANGER sequencing, 2 μL of DNA with concentration of 10 ng/μL was used for amplification. Annealing temperature was set to 55.5°C and 2.5 μM concentration of primers was used. PCR products were subsequently purified using QIAquick PCR purification kit (QIAGEN, Hilden, Germany). SANGER sequencing was performed at EUROFINS Genomics (www.eurofinsgenomics.eu) using ABI 3730xl DNA Analyzer systems.

2.3 | *In silico* functional analysis

Functional analysis was performed using PolyPhen-2 and SIFT tools for prediction of consequences of missense mutations (Adzhubei, Jordan, & Sunyaev, 2013; Adzhubei et al., 2010; Kumar, Henikoff, & Ng, 2009). The mutation was additionally analyzed using MutationTaster (Schwarz, Cooper, Schuelke, & Seelow, 2014). Constrained elements were determined using GERP++ software (Davydov et al., 2010). rsID annotation was carried out using dbsnp human build 150 database. Alignment of protein sequences was carried out using Clustal Omega (Madeira et al., 2019). 3-dimensional structure was obtained from Protein Data Bank 2E9W (Yuzawa et al., 2007) and visualized with CLC Main Workbench 7.7. Hydrophobicity was analyzed using Kyte-Doolitle scale implemented in CLC Main Workbench 7.7 (QIAGEN, Hilden, Germany).

3 | RESULTS

Exome sequencing results revealed a clearly segregated *de novo* common mutation in exon 4 of *KITLG* gene in affected individuals. A NM_000899.4:c.[329A>T] (chr12:88912508A>T) mutation was identified in all three affected individuals. NM_000899.4:c.[329A>T] is leading

to NP_000890.1:p.(Asp110Val). All three affected individuals are heterozygous for NM_000899.4:c.[329A>T]. Other missense mutations in *KITLG* were not detected. Subsequent assessment with SANGER sequencing confirmed the NGS findings (Figure 4). The heterozygous mutation NM_000899.4:c.[329A>T] is clearly identifiable in family members GL2, GL3 and GL4. In unaffected family members, the mutation was not present.

Functional analyses and annotation have shown that the NM_000899.4:c.[329A>T] is a novel substitution in exon 4 of *KITLG*. The substitution is not present in the dbSNP 150 database (www.ncbi.nlm.nih.gov/snp/). Both PolyPhen-2 and SIFT algorithms defined the substitution as probably damaging (score: 0.999) and deleterious, respectively. In addition, MutationTaster identified the substitution as disease causing with 99% probability. Furthermore, the substitution is located in a conserved region with GERP++ score of 5.95. Additionally, the conserved region was confirmed also with alignments of KITLG protein sequences of different species (Figure 5). The substitution is located on the 3rd alpha helix of the KITLG protein (Figure 6). Hydrophobicity analysis has shown that mutant version of the KITLG protein has increased hydrophobicity at the mutation site (1.4 vs. 2.2 for the mutant).

4 | DISCUSSION

Here we present a pathogenic *de novo KITLG* gene mutation leading to NP_000890.1:p.(Asp110Val) substitution segregated in a Slovenian family with history of unique pigmentary abnormalities representing a variant of FPHH and colorectal cancer. The pigmentary abnormalities previously not described in FPHH families include periorbital hyperpigmentation, hypopigmentation within hyperpigmented lesions (eg. CALS) and hyperpigmentation within hypopigmented lesions. The mutation

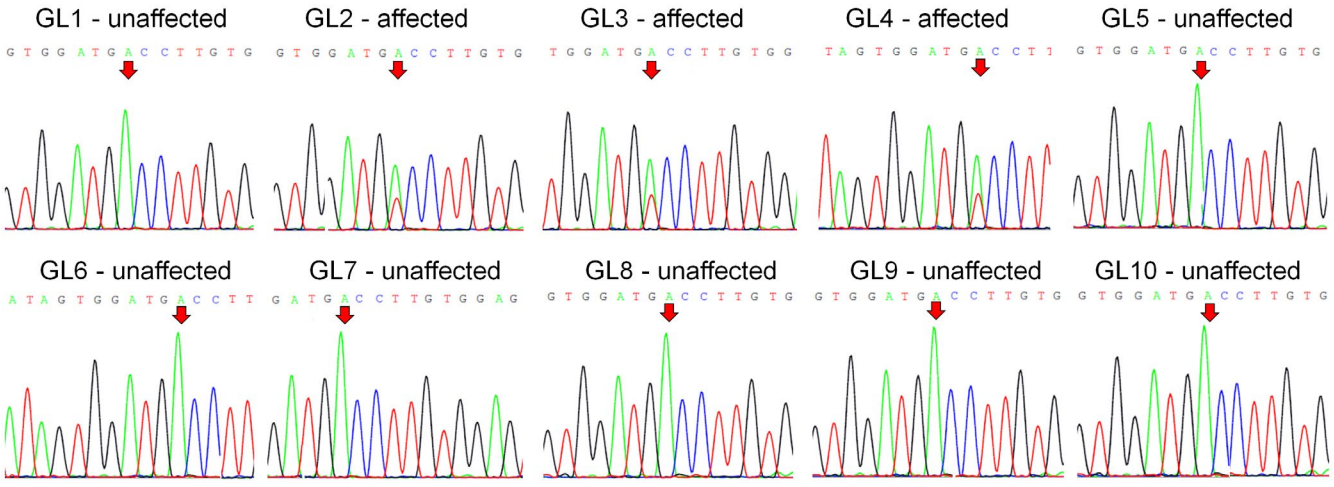

FIGURE 4 Family screening for NM_000899.4:c.[329A>T] through SANGER sequencing. Arrow indicates the mutation position

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                                        D110V
MUTATION            KFSNISEGLSNYSIIDKLVNIVDVLVECVKENSS   120
Rattus norvegicus1  KFSNISEGLSNYSIIDKLGKIVDDLVACMEENAP   120
Rattus norvegicus2  KFSNISEGLSNYSIIDKLGKIVDDLVACMEENAP   120
Homo sapiens a      KFSNISEGLSNYSIIDKLVNIVDDLVECVKENSS   120
Homo sapiens b      KFSNISEGLSNYSIIDKLVNIVDDLVECVKENSS   120
Sus scrofa          KFSNISEGLSNYSIIDKLVKIVDDLVECMEEHSF   120
Ovis aries          KFSNISEGLSNYSIIDKLVKIVDDLVECMEEHSF   120
Bos taurus          KFSNISEGLSNYCIIDKLVKIVDDLVECMEEHSS   120
Capra hircus        KFSNISEGLSNYSIIDKLVKIVDDLVECMEEHSF   120
Canis lupus fam.    KFSNISEGLSNYSIIDKLVKIVDDLVECTEGYSF   120
Felis catus         KFSNISEGLSNYSIIDKLVKIVDDLVECVEGHSS   120
                    ***********.***** :***M** * :  :
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FIGURE 5 Alignment of KITLG protein sequences. (*) indicates complete alignment; (.) indicates misalignment of one sequence; (:) indicates misalignment of two sequences; () indicates misalignment

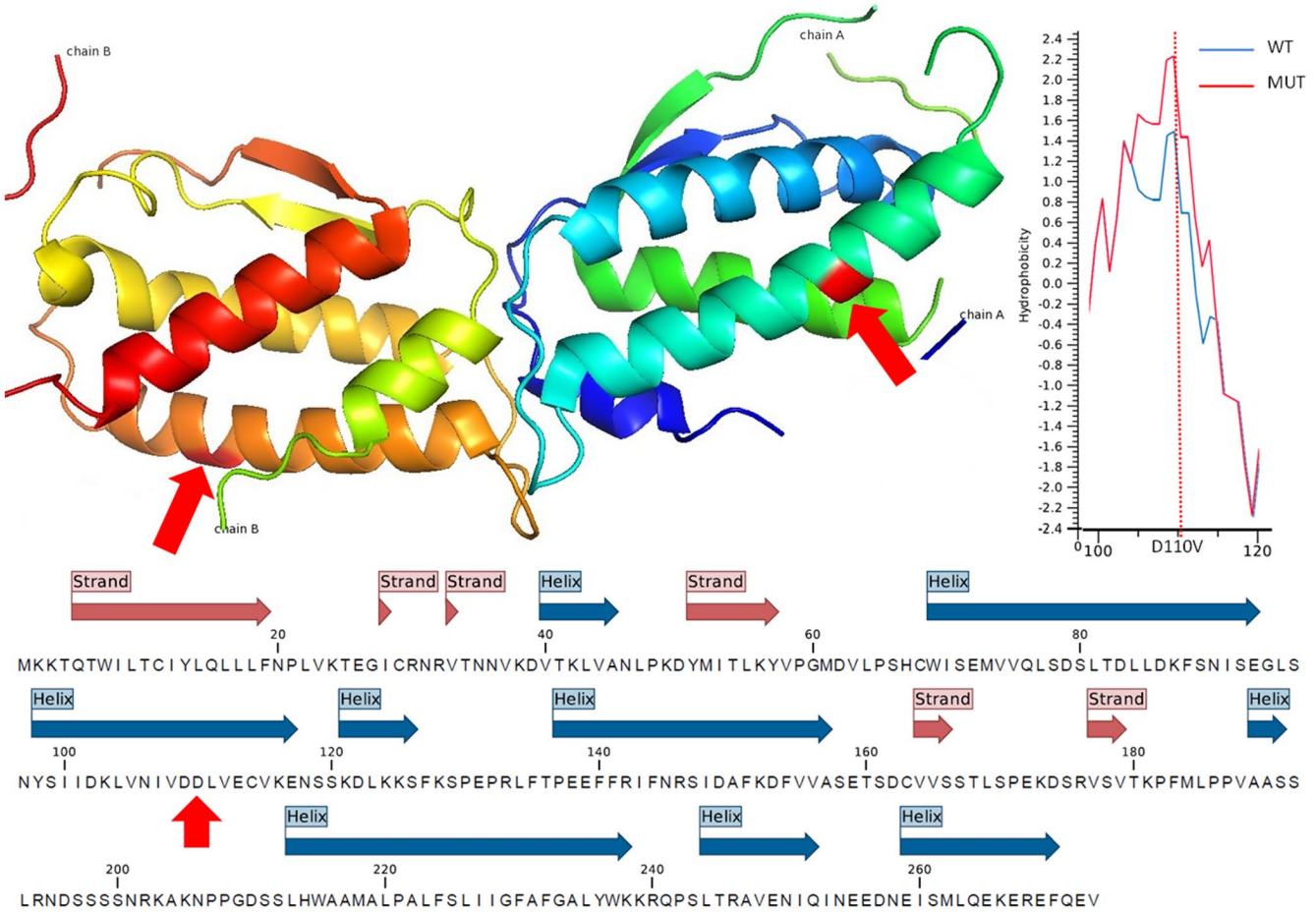

FIGURE 6 Location of *de novo* NP_000890.1:p.(Asp110Val) substitution in a 3D KITLG model and hydrophobicity analysis plot. Red arrows are indicating the mutation site. Blue (WT) line is for wild type protein and red (MUT) line is for mutant protein

perfectly segregated within family and was not detected in healthy family members, thus providing evidence that NM_000899.4:c.[329A>T] underlies dominantly inherited variant of FPHH. Furthermore, we provide additional evidence of likely association of the aforementioned substitution with colorectal cancer.

Melanoblast development, differentiation, melanosome formation and synthesis of melanin is influenced by many factors and mutations affecting these processes can result in hereditary hypomelanoses (Amyere et al., 2011). Some of the known involved factors are c-Kit receptor and its ligand KITLG. After KITLG binds to the c-Kit receptor, ligand-induced dimerization is triggered (Lev, Yarden, & Givol, 1992), and intracellular tyrosine kinase is activated (Spritz, 1994). This results in the autophosphorylation of tyrosine residues within the KIT kinase domain, enhancing the binding of various proteins, which act as mediators of mitogenic signal transduction in the KIT-dependent pathway (Morrison-Graham & Takahashi, 1993). Previous evidence suggests that decreased KIT-dependent signal transductions is involved in the abnormal distribution and decreased proliferation of melanoblasts during embryologic development (Spritz, 1994; Spritz, Ho, & Strunk, 1994). Mutations in different proteins involved in this pathway also cause pigmentation disorders such as neurofibromatosis type 1 characterized by multiple café-au-lait spots (De Schepper et al., 2008), Legius syndrome (Brems et al., 2007) and piebaldism (Spritz, 1994). Thus, evidence pinpoints to a pivotal role of the KITLG/c-Kit signaling pathway in human skin determinism (Amyere et al., 2011). Eight previously identified substitutions in KITLG were associated with FPHH and FPH (Amyere et al., 2011; Cuell et al., 2015; Wang et al., 2009; Zhang et al., 2016). Among these, seven were found to be located in a highly conserved VTNN motif and one within another ligand-receptor interaction site. Here we studied a Caucasian family with a clearly segregated *de novo* NM_000899.4:c.[329A>T] mutation located in *KITLG* exon 4 leading to NP_000890.1:p.(Asp110Val) substitution also located within another highly conserved ligand-receptor interaction site. In comparison to previously published cases of FPHH, we noticed more pronounced diffuse hyperpigmentation in the present pedigree. *KITLG* exon 4 encodes a part of extracellular domain, which was identified as the functional core of the ligand for

binding and subsequent activation of the receptor (Langley et al., 1994). Two of the *N*-linked glycosylation sites and two of the cysteine residues taking part in disulfide bonds are located in exon (Hadjiconstantouras et al., 2008). *In silico* functional analyses have shown that the NP_000890.1:p.(Asp110Val) substitution is predicted pathogenic by SIFT and PolyPhen-2 and it was flagged as disease causing substitution located in a highly conserved region on 3rd alpha helix of the KITLG protein. *In silico* hydrophobicity analysis has also shown an increase in hydrophobicity in the mutant protein suggesting profound changes of the protein features which can in turn impact its affinity to c-Kit. It is known and widely believed that hydrophobic effect is the main driving force behind the folding of proteins (Dill, 1990). Furthermore, the hydrophobic effect is associated with protein behaviors as revealed through water-protein analyses of hydrogen bonds (Camilloni et al., 2016). In contrast to our findings, the only known substitution outside VTNN motif causing FPHH, NP_000890.1:p.(Glu113Lys), was not predicted to be pathogenic by SIFT and PolyPhen-2 algorithms (Kato et al., 2020). Furthermore, NP_000890.1:p.(Glu113Lys) is located in a lesser conserved region in comparison to NP_000890.1:p.(Asp110Val). Moreover, in contrast with patients with kidney or colon cancer, in whom pathogenic mutations in KITLG are located in alpha helix, seven previously published FPHH related mutations are located within VTNN motif outside of the somatic mutation region in alpha helix (Cuell et al., 2015). This in turn may implicate coincidental findings of cancers and FPHH associated VTNN mutations. Using Catalogue of Somatic Mutations in Cancer (COSMIC: cancer.sanger.ac.uk/cosmic) we observed that in the 3rd alpha helix (aa.99–117) somatic pathogenic mutations caused large intestinal adenocarcinoma NP_000890.1:p.(Leu104Pro), skin basal cell carcinoma NP_000890.1:p.(Leu111Phe) and lung adenocarcinoma NP_000890.1:p.(Val115Leu) (McMillan et al., 2018; Mouradov et al., 2014; Sharpe et al., 2015). Based on that, malignancy found in proband GL2 may not be just coincidental with *de novo* germline mutation found in IVDDLV motif, but may be associated with NP_000890.1:p.(Asp110Val) mutation causing FPHH. Thus, the present study provides important additional evidence of association of the *KITLG* mutation with both FPHH and malignancy.

Interestingly, a study studying Dutch and Spanish families identified additional mutations in *KITLG* (NP_000890.1:p.(His67_Cys68delinsAr), NP_000890.1:p.(Ser96Ter), NP_000890.1:p.(Leu104Val)) which were segregated in families with non-syndromic unilateral and asymmetric hearing loss and Waardenburg syndrome type 2, demonstrating that mutations in *KITLG* can underlie different phenotypes, depending on the effect of mutation (loss-of-function, dominant-negative, dominant-activting) (Zazo Seco et al., 2015). Previous evidence shows that FPHH causing KITLG mutations are gain-of-function mutations (Amyere et al., 2011; Wang et al., 2009). Hence, NP_000890.1:p.(Asp110Val) likely causes gain-of-function mutation underlying a variant of FPHH. We hypothesize that increased hydrophobicity might be the underlying mechanism affecting the affinity to c-Kit and causing a gain-of-function mutation effect. However, literature about FPHH is scarce and discrepancies in phenotype descriptions exist.

Difficulties arose with classification of our patients into existing diagnosis due to unique phenotype features and due to inconsistency in the published literature. Initially, *KITLG* mutations were reported in Chinese families described as familial progressive hyperpigmentation (FPH) (Wang et al., 2009). Later, the *KITLG* mutations were identified within Familial Progressive Hyper- and Hypopigmentation families (FPHH) and suggested FPHH is distinct from FPH, in which no hypopigmented features are present, and which is phenotypically and histologically closer to DUH2 (Amyere et al., 2011). Recently, FPH was joint together with FPHH under new OMIM diagnosis "Familial Progressive Hyper- with or without Hypopigmentation" (FPHH, MIM: 145250), possibly to distinguish FPH families with proven *KITLG* mutations from families with familial progressive hyperpigmentation (FPH1, MIM: 614233) that were mapped to Chr 19pter-p13.1 region. The new designed diagnosis "Familial progressive hyper- with or without hypopigmentation" (FPHH, MIM: 145250) is currently used in OMIM and ClinVar databases, but was so far to the best of our knowledge used in the literature only once and inconsistently by Kato et al. (2020), where diagnosis "Familial Progressive Hyper- with or without Hypopigmentation" was used in the title and as a keyword, but diagnosis "Familial Progressive Hyper- and Hypopigmentation" was used in the abstract and in the text for the same patients within this study. To avoid further confusion, we propose to classify our family as a new variant of Familial Progressive Hyper- and Hypopigmentation, and thus a third subtype in addition to FPH and classic FPHH, within broader diagnosis "Familial Progressive Hyper- with or without Hypopigmentation" (FPHH, MIM: 145250). We propose that "Familial progressive hyper- with or without hypopigmentation" (FPHH) is divided into 3 subtypes that are all characterized by *KITLG* mutations, share similar phenotype, but are distinguished by specific clinical manifestations described as follows: I. FPHH, subtype 1: Familial Progressive Hyper- and Hypopigmentation (FPHH): (Hyper- and Hypopigmentation lesions); II. FPHH, subtype 2: Familial progressive hyperpigmentation (FPH): (Hyper- but no Hypopigmentation lesions); III. FPHH, subtype 3: A variant of FPHH (our family): (Hyper- and Hypopigmentation lesions plus additional lesions:

periorbital hyperpigmentation, hypopigmentation within hyperpigmented lesions (eg. CALS) and hyperpigmentation within hypopigmented lesions; optional: cancer (colorectal) comorbidity).

Since *de novo mutation* identified in this study was located in different *KITLG* gene region than previous *KITLG* mutations reported in FPHH, subtype 1 and FPHH, subtype 2 families, it is also possible, that subtype 3 identified by our study, could be distinguished also genetically from subtype 1 and 2 by location of the mutation within specific *KITLG* gene region (coding for 3rd alpha helix domain), however this needs to be confirmed in further studies when new families with the same subtype 3 phenotype will be identified and genetically characterized.

In summary, our findings clearly confirm an additional hot spot of *KITLG* mutations in the 3rd alpha helix, which might also increase the risk of malignancies in FPHH. Thus, our findings could have a pivotal relevance in clinical settings for the patients presenting with FPHH suggesting that FPHH diagnosed patients should subsequently undergo preventive screenings in order to prevent malignancies.

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CONFLICT OF INTEREST

The authors declare no competing interests or conflict of interest. No benefits in any form have been received from any commercial party.

AUTHOR CONTRIBUTIONS

Conceptualization, M.G, N.F., P.B.M. and U.P.; methodology, M.G.; investigation, M.G, N.F., P.B.M., M.Ž. and U.P.; data curation, M.G, N.F., P.B.M., M.Ž. and U.P.; writing—original draft preparation, M.G, N.F., P.B.M., M.Ž. and U.P.; writing—review and editing, M.G, N.F., P.B.M. and U.P.; visualization, M.G. and N.F.; supervision, U.P.; All authors have read and agreed to the published version of the manuscript.

DATA AVAILABILITY STATEMENT

Data available on request due to privacy/ethical restrictions.

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