

Original Article

A novel c.2326G>A KIT pathogenic variant in piebaldism

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Received April 22, 2020; Accepted September 9, 2020; Epub October 15, 2020; Published October 30, 2020

Abstract: Introduction: Piebaldism is a rare autosomal dominant disorder characterized by congenital patchy depigmentation of the scalp, forehead, trunk, and limbs. The *KIT* gene is the mainly causative gene to this disease. But how *KIT* is involved in piebaldism remains unclear. Methods: Whole exome sequencing was used to explore the genetic cause of a familial case of piebaldism. Sanger sequencing was used to validate the variant. To further examine the variant's pathogenicity, the wild type and the mutated *KIT* plasmids were constructed and transfected into HEK293T cells. Next STAT5 expression, a signaling target of KIT, was detected by western blotting to explore the potential molecular mechanism of the variant in piebaldism. Based on the classification of the given variant, prenatal diagnosis was further performed in this family. Results: A novel pathogenic variant of *KIT* c.2326G>A (NM_000222.2) was identified in this family. The phosphorylation of STAT5 was reduced in the mutant *KIT* transfected cells compared to the wild type after stem cell factor (SCF) treatment, indicating that the KIT signaling was dysfunctional and supported that the variant was a pathogenic one. Prenatal diagnosis results indicated that the fetus exhibited the same genotype as the proband. Conclusion: We identified a novel *KIT* pathogenic variant in the patient with piebaldism to expand the variation spectrum of *KIT*. The functional study indicated that the mutant KIT was dysfunctional in KIT signaling. The pathogenic variant identification enriches the knowledge about the genotype/phenotype correlation and could serve as the basis for genetic counseling and prenatal diagnosis.

Keywords: Piebaldism, *KIT*, novel variant, STAT5, prenatal diagnosis

Introduction

Piebaldism (MIM# 172800) is a rare autosomal dominant disorder characterized by variable patches of depigmentation on the scalp, forehead, trunk, and limbs [1-3]. It is suggested that piebaldism is due to aberrant proliferation or migration of neural crest-derived melanoblasts in the embryo [1]. Mutations in the *KIT* gene (MIM# 164920) have been identified as the most common cause of human piebaldism [4].

The *KIT* gene encodes the KIT protein, a 145-kD glycosylated transmembrane protein, which also classified as a class III tyrosine kinase receptor. The KIT receptor is expressed in melanoblasts and differentiated melanocytes, which

are involved in piebaldism [5]. The receptor consists of three domains: an extracellular ligand-binding domain, a transmembrane domain, and a cytoplasmic region. The cytoplasmic region has highly conserved tyrosine-specific kinase activity. It can be further divided into a juxtamembrane domain and a TK domain which is further subdivided into a TK domain 1 (TK1) (amino acid residues, 582-684), a kinase insert domain (amino acid residues, 685-761), and a TK domain 2 (TK2) (amino acid residues, 762-973). The KIT is homodimerized after binding to its ligand, stem cell factor (SCF), and its intrinsic kinase activity is activated via autophosphorylation of its tyrosine residues. The different tyrosine phosphorylation sites interact with multiple downstream signaling pathways, including the PI3K [6], MAPK [7], Jak/Stat [8],

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and Src family kinase pathways [9]. The resultant signals are involved in the regulation of cell growth, survival, migration, and differentiation [8]. The clinical features and severity of piebaldism correlate with the site and type of mutation in the *KIT* gene [10]. It was reported that the pathogenic variants in the intracellular tyrosine kinase domain resulted in a severe manifestation of piebaldism [3].

To date, more than 70 mutations of *KIT* have been identified, but the functions of the mutant *KIT* proteins are largely unknown. In the present study, we identified a novel pathogenic variant of the *KIT* gene in a familial case of piebaldism. Then we performed *in vitro* experiments to study the function of the mutant *KIT* protein. Finally, prenatal diagnosis was carried out based on the pathogenic variant in this family.

Material and method

Ethics statement

Ethical approval was obtained from the Ethics Committee of Henan Provincial People's Hospital, China. All individuals involved in this study agreed to the research plan and signed the consent document, or informed consent was obtained from parents for the children under the age of 18 years.

Patients

The proband is a 26-year-old woman. She has sections of white hair near her front hairline and patches of unpigmented skin on her forehead, abdomen, arms, and legs. The inner third of her eyebrows also is white. The unpigmented areas were present since birth and did not increase in size or number. Besides, the unpigmented skin is prone to redness and sunburn. Based on the clinical classification, the proband was defined as exhibiting a severe form of piebaldism. Her mother and her maternal uncle have a similar phenotype. The amniotic fluid sampling under ultrasonic guidance was performed for the proband at 19 weeks of pregnancy.

Whole exome sequencing

Genomic DNA of the patients was captured via a SureSelect Human All Exon V5+UTR kit (Agilent Technologies, California). Next, exon-enriched DNA libraries were sequenced on an

Illumina HiSeq2000 system (Illumina, California) using 100 bp pair-end reads. High-quality reads were aligned to the human reference genome GRCh37. Broad Institute Genome Analysis Tool Kit (GATKv2.6) was used for variant calling. ANNOVAR 11 and SnpEff were used for variant annotation. All exome variants were filtered for allele frequencies less than 0.001 in gnomAD database. The final candidate variant was further confirmed by Sanger sequencing.

Sanger sequencing

PCR amplification products were sequenced by Sangon Biotech Co. Ltd (Shanghai, China) to analyze the given variant. Primers were designed as follows: *KIT*, Forward: 5'-TGGCAGTTA-GGGTTGCAAGT-3', Reverse: 5'-GAGACAGCAGT-TGGAACATGA-3'.

Plasmid construction

Wild type and mutant *KIT* genes were amplified by PCR, then cloned into pcDNA3.1 vector (Invitrogen, USA) via Seamless Cloning Kit (Hanbio, China). The wild type and mutant plasmid were designated as pcDNA3.1-KIT-wt and pcDNA3.1-KIT-mut respectively. The sequence was validated via Sanger sequencing.

Cell culture, transfection and protein extraction

Human embryonic kidney 293T cells (HEK293T) were maintained at 37°C and 5% CO₂ in a high glucose formulated DMEM (Solarbio, China) supplemented with 10% fetal bovine serum. HEK293T cells were plated in 6-well plates overnight before transfection. The pcDNA3.1-KIT-wt and pcDNA3.1-KIT-mut were transfected into HEK293T cells respectively using Lipofectamine 2000 reagent (Invitrogen, USA) according to the manufacturer's instructions. Forty-eight hours later, the cells were treated with 50 ng/ml stem cell factor (Solarbio, China) for 15 minutes, then lysed with RIPA (Solarbio, China), supplemented with protease inhibitor cocktail (Roche, Indianapolis, USA). The cells were collected and centrifuged at 12000 rpm for 15 minutes at 4°C. The supernatant was collected and used for western blotting.

Western blotting

Western blotting was performed as described previously [11]. Specifically, proteins were sep-

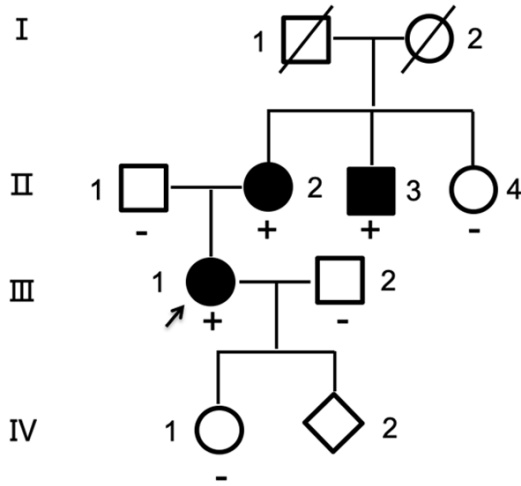


Figure 1. Pedigree of the family. The pedigree is shown with squares representing males, circles representing females. Affected individuals are indicated in blackened symbols, and open symbols represent unaffected persons. III-1 is the proband marked with a black arrow. Genotype of each individual is exhibited with +/- . + indicates the individual with the given variant *KIT* c.2326G>A, while - indicates the individual without the given variant.

arated on SDS-PAGE gels. Antibodies for detecting protein were diluted at 1:1000 for anti-STAT5 (D206Y) antibody (Cell Signaling, USA), an anti-phospho-STAT5 (D47E7) antibody (Cell Signaling, USA) and 1:5000 for anti- β -ACTIN (13E5) antibody (Cell Signaling, USA). The HRP-labeled secondary antibody (Cell Signaling, USA) was diluted at 1:10,000 and detected with enhanced chemiluminescence, NcmECL Ultra kit (NCM Biotech, China).

Maternal cell contamination detection in amniotic fluid

In order to test if there was the maternal cell contamination in the amniotic fluid, DNA samples from the pregnant woman's blood and her amniotic fluid were amplified using PowerPlex 21 HS genotyping system (Promega, Madison) according to manufacturer's instructions. Amplification was performed in a volume of 25 μ l, including 5 μ l of PowerPlex 21 5 \times Master Mix, 5 μ l of PowerPlex 21 5 \times Primer Pair Mix and 5 ng of DNA. The program is as follows: 96 $^{\circ}$ C for 1 minute, 94 $^{\circ}$ C for 10 seconds, 59 $^{\circ}$ C for 1 minute, 72 $^{\circ}$ C for 30 seconds, 30 cycles in total; 60 $^{\circ}$ C for 10 minutes.

Results

The novel KIT variant was identified in the family

The pedigree of this family is in **Figure 1**. In the family, we identified a novel heterozygous missense variant c.2326G>A (p. Val776Met) in the *KIT* gene (NM_000222.2) in the proband, her mother, and her maternal uncle via whole-exome sequencing analysis, which was confirmed through Sanger sequencing. The variant was absent in the proband's father, aunt, husband, and daughter. The Sanger sequencing confirmation of the *KIT* variant is shown in **Figure 2**. By PolyPhen-2, this variant was predicted as probably damaging. By SIFT, this variant was predicted to be damaging. The allele frequency of this variant is zero in gnomAD exome and gnomAD genome database. All of these results support that this variant probably has pathogenic significance.

The phosphorylation of STAT5 reduced in the mutant KIT-expressing HEK293T cells after SCF stimulation

The schematic diagrams of the *KIT* mRNA and protein are shown in (**Figure 3A, 3B**). Comparative analysis of the amino acid sequences of KIT from human, Rhesus, mouse, dog, elephant, chicken, tropicalis, zebrafish, and lamprey showed that the residue V766 is highly conserved (**Figure 3C**). The variant is located in Exon 16, which encodes the TK2 domain. Since the JAK/STAT signaling pathway (**Figure 3D**) could be activated by KIT activation and the STAT will be further phosphorylated to exert its function [8, 12], we investigated whether the mutation impaired the KIT biological function in this signaling pathway. After SCF stimulation, the wild type and mutated KIT-transfected cells were harvested, and the total and phosphorylated STAT5 were detected using Western blotting. The phosphorylation of STAT5 was detected in wild type *KIT* transfected cells but dramatically reduced in mutant transfected cells, while no obvious change was observed between two groups for the total STAT5 expression (**Figure 3E**). This result indicates that the mutant KIT is dysfunctional. This result supports that *KIT* c.2326G>A was classified as a pathogenic variant according to ACMG guideline (PS3; PM2; PP1_Moderate; PP3; PP4).

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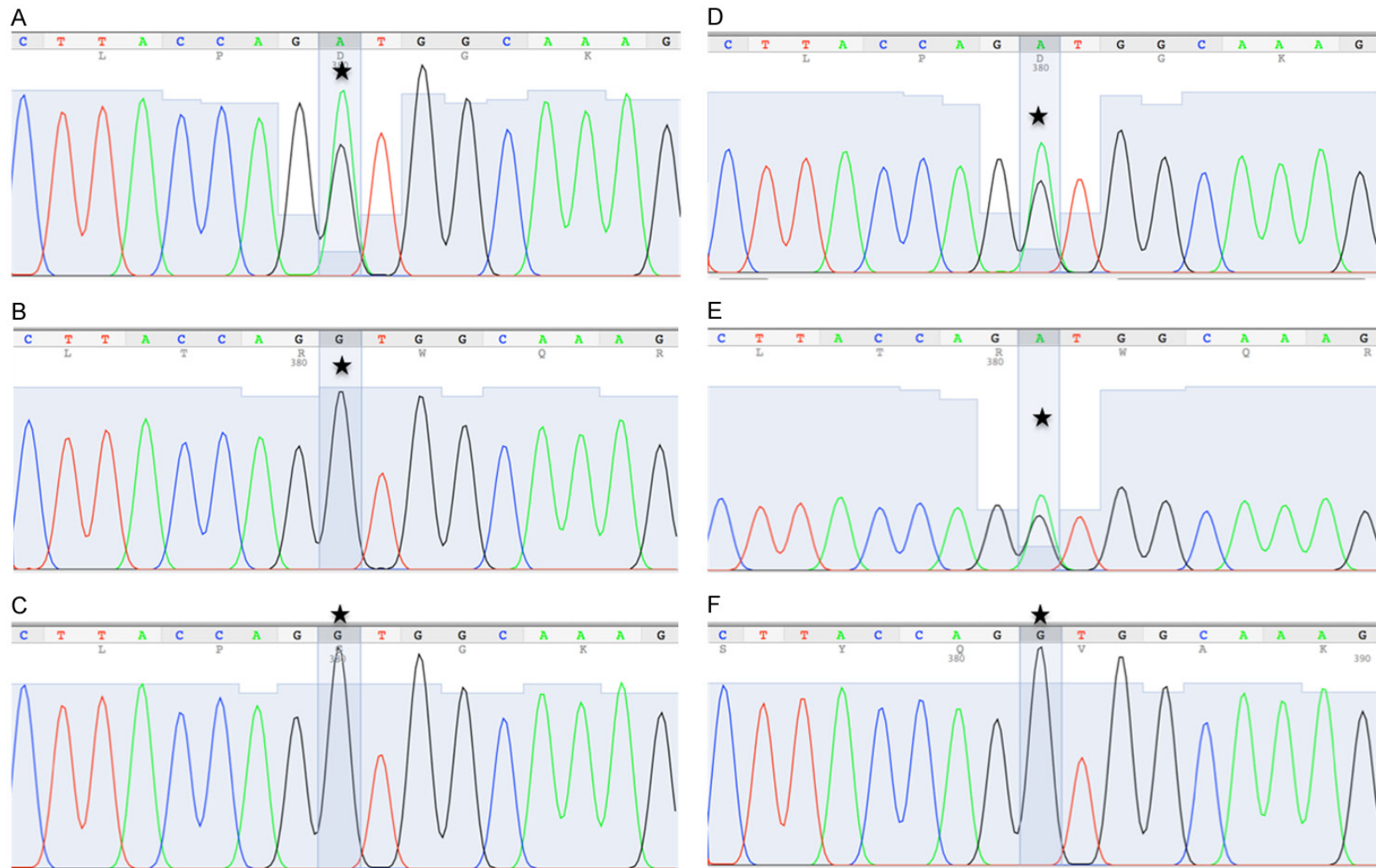


Figure 2. Sanger sequencing results of the family. The heterozygous status of c.2326G>A in the KIT gene was confirmed by Sanger sequencing in the proband (A), proband's mother (D) and maternal uncle (E). The proband's father (B), husband (C) and daughter (F) showed a wild type genotype. The position of the variant or the corresponding wild type nucleotide is labeled with a black asterisk (*).

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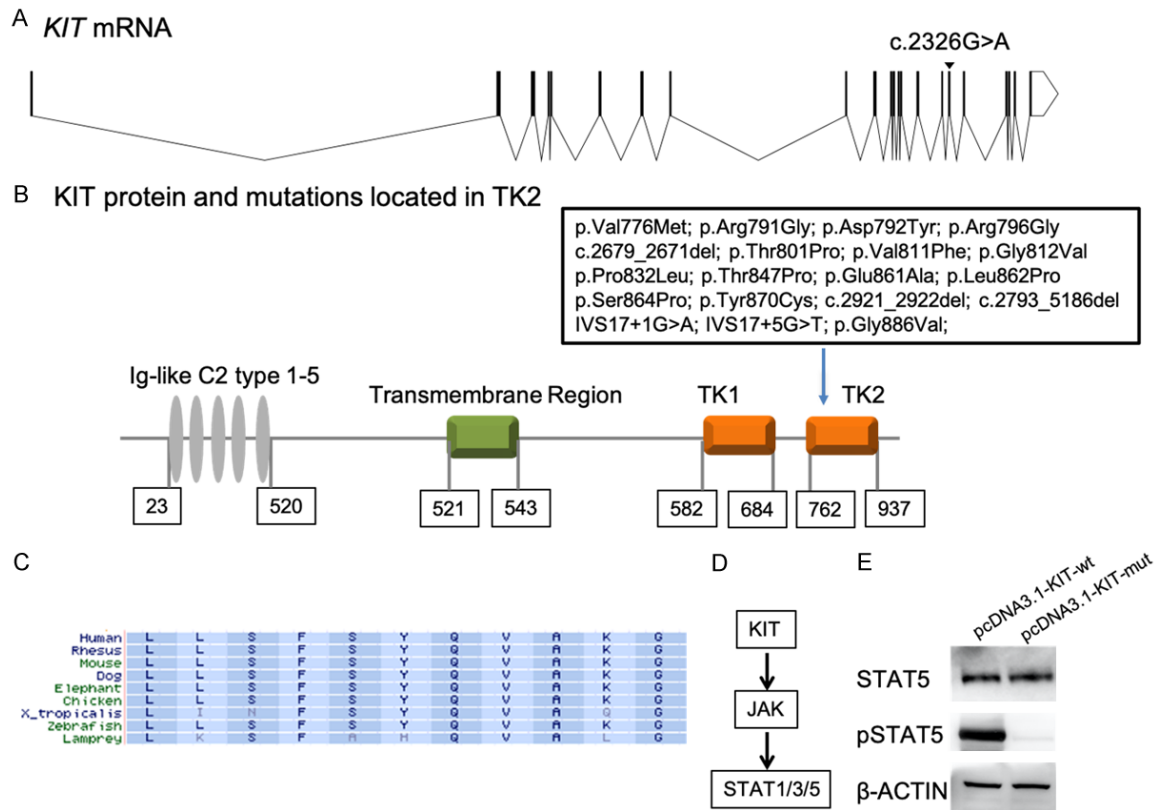


Figure 3. Molecular characterization of the variant *KIT* c.2326G>A. A. Schematic structure of *KIT* gene illustrating the variant. Exons are shown as boxes and introns as lines. The black inverted triangle indicates the location of the variant. B. Schematic representation of the KIT protein and summary of the pathogenic variants located in the TK2 domain. The black box indicates the pathogenic variants including p.Val776Met located in the TK2 domain. C. KIT protein sequences from different organisms. The V776 in KIT is highly conserved between human, Rhesus, mouse, dog, elephant, chicken, tropicalis, zebrafish, and lamprey. Amino acid region 769-779 of human protein sequences are shown. D. KIT activates JAK/STAT signaling pathway after SCF stimulation. E. Expression of STAT5 and Phosphorylated STAT5 after SCF stimulation. Immunoblot analysis showed that the phosphorylation of STAT5 was reduced in mutant *KIT* transfected cells compared to wild type *KIT* transfected cells. β -ACTIN served as an internal control. pSTAT5, the phosphorylation of STAT5.

Prenatal diagnosis in this family

Prenatal diagnosis was carried out after identification of the causative variant to piebaldism in the family. Maternal contamination was excluded in the DNA from the amniotic fluid (Figure 4), then the genotype of the given location in the gene of *KIT* was tested. The fetus was found to be heterozygous for c.2326G>A pathogenic variant (Figure 5) and therefore predicted to exhibit piebaldism.

Discussion

Most of piebaldism is caused by a loss-of-function variant in the *KIT* gene. In this study, we identified a novel pathogenic variant c.2326G>A in the *KIT* gene in a familial case of

piebaldism in the inheritance pattern of autosomal dominant.

The *KIT* gene encodes the cell surface transmembrane tyrosine kinase receptor, KIT. As a receptor, KIT contains five extracellular immunoglobulin-like domains, which are essential for SCF binding. The tyrosine residues autophosphorylation in the TK domain in the cytoplasmic region activates its intrinsic kinase activity in response to SCF binding [13]. KIT has multiple tyrosine phosphorylation sites, which interact with subsequent signaling pathways, such as JAK/STAT [12]. In this study, since the Val776Met was localized in the intracellular tyrosine kinase region, the mutant KIT protein probably still can bind SCF. But SCF-induced phosphorylation of STAT5 was dramatically

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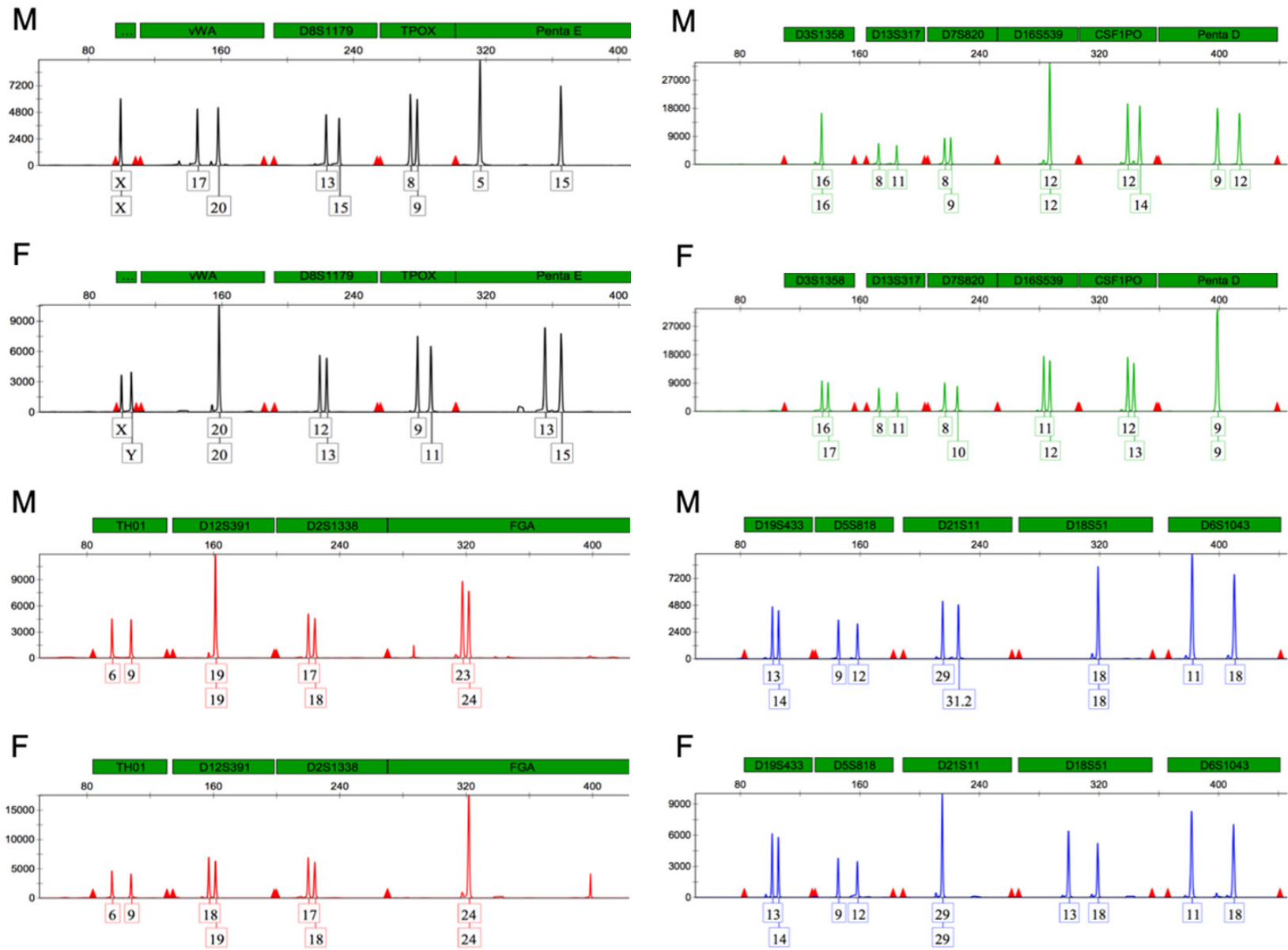


Figure 4. Exclusion of maternal contamination in the amniotic fluid. Twenty-one STR loci including D1S1656, D2S1338, D3S1358, D5S818, D6S1043, D7S820, D8S1179, D12S391, D13S317, D16S539, D18S51, D19S433, D21S11, Amelogenin, CSF1PO, FGA, Penta D, Penta E, TH01, TPOX, and vWA were analyzed. (M) STR profile of the proband; (F) STR profile of the fetus.

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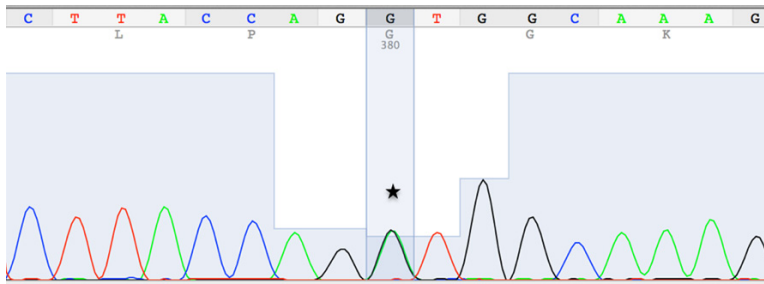


Figure 5. The genotype at the given locus in the fetus. The fetus shows the heterozygous status of c.2326G>A in the *KIT* gene. The position of the pathogenic variant is labeled with a black asterisk (*).

reduced in mutant *KIT*-expressing HEK293T cells, which indicated that the mutant *KIT* was dysfunctional in *KIT* signaling. This result is consistent with another pathogenic variant *KIT* c.645_650delTGTGTC published elsewhere [14], which also exhibited reduced *KIT* activity.

The defective proliferation or migration of melanocytes from the neural crest during development is highly related to piebaldism [10]. It has been reported that the RAS/MAPK cascade activated by SCF/*KIT* plays essential roles in melanocyte migration, melanogenesis, and melanosome transfer [13, 15]. In the present study, we identified a variant c.2326G>A in the *KIT* gene affecting the phosphorylation of STAT5, resulting in piebaldism. But whether the variant is related to abnormal proliferation or migration of melanocytes in the lesions of carriers needs further study.

We also carried out a prenatal diagnosis for this family based on the identified pathogenic variant *KIT* c.2326G>A. After excluding the maternal contamination in the amniotic fluid, the fetus showed the same genotype to the proband. This result indicated that the fetus would probably have the same phenotype as the proband. The follow-ups are still in process.

Conclusions

In conclusion, a novel pathogenic variant c.2326G>A in the *KIT* gene was identified in this familial case of piebaldism. Functional studies showed that the mutant *KIT* reduced the phosphorylation of STAT5, which indicated that the mutant *KIT* was dysfunctional in *KIT* signaling. This study enriches our knowledge about the genotype/phenotype correlation, although the variant's pathogenicity still needs

further elucidation. Further investigation could offer strategies for novel treatments for piebaldism as well as other pigmentary disorders. The study also serves as a basis for genetic counseling and prenatal diagnosis in a clinical setting.

Acknowledgements

We thank the patients and their families for their participation in this study. Also, we thank all the members of our lab for their discussions on this project. This study was supported by grants from National Natural Science Foundation of China (81650010, 31670892, and 31970836), Henan Province Health System Abroad Training program (2016059), and Open Research Fund from National Health Commission Key Laboratory of Birth Defect Prevention (ZD202005).

Disclosure of conflict of interest

None.

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