A *KRT6A* Mutation p.Ile462Asn In A Chinese Family With Pachyonychia Congenita, And Identification of Maternal Mosaicism

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Research Article

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Abstract

Background: Pachyonychia congenita (PC, OMIM #167200, #167210, #615726, #615728, and #615735) is a rare autosomal dominant disorder caused by keratin gene mutations in *KRT6A*, *KRT6B*, *KRT6C*, *KRT16* or *KRT17*. It is characterized with nail dystrophy and palmoplantar keratoderma (PPK). The most prominent manifestation is plantar pain. This is the first reported case of maternal mosaicism in PC. Although very rare, germ cell mosaicism should be considered when providing genetic counselling for unaffected parents of a child with PC.

Methods: Genomic DNA was extracted from peripheral blood samples, hair bulbs, buccal smears and the father's germ cells. The entire coding and flanking intronic sequences of 5 keratin genes were screened for mutations in every individuals of the family by Sanger sequencing. We used whole exome sequencing (WES) to search for mosaicism in the parents who had no *KRT6A* mutation identified by Sanger sequencing. Mosaicism was confirmed by SNaPshot sequencing and HiSeq deep sequencing.

Results: A previously reported heterozygous mutation, p.Ile462Asn, was identified in KRT6A in the proband and his affected sister. The variant was detected in one sequencing read from 86 sequencing reads from DNA from the mother's blood by WES. The mutation was not identified in DNA from the father's blood. Frequency of reads was 47% and 49% in proband and his sister, respectively. SNaPshot sequencing revealed mosaicism at a level of 2.5% and 4.7% in DNA from blood and hair bulbs from the unaffected mother. HiSeq deep sequencing demonstrated low-grade mosaicism in the patient's younger sister and parents.

Conclusion: These findings indicate the ability of WES and SNaPshot sequencing to detect low-frequency mosaic mutations. Although very rare, germinal mosaicism should be considered when genetic counseling is given to families with presumed spontaneous cases of PC.

Background

Pachyonychia congenita (PC, OMIM #167200, #167210, #615726, #615728, and #615735) is a rare autosomal dominant disorder caused by keratin gene mutations in *KRT6A, KRT6B, KRT6C, KRT16 or KRT17* [1]. It is characterized with nail dystrophy and palmoplantar keratoderma (PPK). The most prominent manifestation is plantar pain [2]. Additional characteristics can include oral leukokeratosis, epidermal inclusion cysts, pilosebaceous cysts, follicular keratoses, hyperhidrosis and sometimes natal teeth [3].

Although very rare, germline mosaicism has been confirmed by molecular diagnosis for some dominant diseases, including keratin disorders epidermolysis bullosa simplex (EBS) and PC as well as dystrophic epidermolysis bullosa pruriginosa, and Ehlers-Danlos syndrome type IV [4, 5, 6]. In 2011, Pho et al. reported the first case of germ cell mosaicism in PC [7]. There were two affected children with unaffected parents in this family. The authors confirmed the pathogenic mutation p.Asn172del in *KRT6A* gene from the unaffected father's sperm cells. To date (April 2021), there are more than 1038 PC patients in the

International Pachyonychia Congenita Research Registry (IPCRR, www.pachyonychia.org), that have genetically confirmed PC, this is the only family with germ cell mosaicism. Gu et al. reported a Japanese EBS patient with a de novo 1649delG mutation in *KRT5* gene in 2003 [8]. The parents were unaffected and the mutation was not detected in DNA derived from blood samples therefore it was reported as a de novo mutation [9]. However, the proband's younger sister was revealed to be affected with EBS at birth in 2004. Further investigations demonstrated somatic and germline mosaicism in the mother of two affected children [5].

In this study, we identified a KRT6A mutation, p.Ile462Asn, in a Chinese PC family with two affected children with unaffected parents. Using whole exome sequencing (WES) and SNaPshot sequencing we confirmed inheritance by maternal mosaicism. To our knowledge, this is the first family of maternal mosaicism in PC.

Methods

DNA extraction

After informed consent, genomic DNA was extracted from the peripheral blood lymphocytes of this family. DNA was also extracted from hair bulbs, buccal smears and sperm cells of the proband's father and hair bulbs and buccal smears of his mother using a QIAGEN QIAamp Blood Mini kit. This study was approved by the Ethics Committees of Shanghai Jiaotong University School of Medicine and conducted in accordance with the principles of the Declaration of Helsinki.

PCR and Sanger sequencing

We analyzed KRT6A, KRT6B, KRT6C, KRT16 and KRT17 genes of this family by direct sequencing using primers and reaction conditions as previously described. In addition, samples from 100 unrelated population-matched controls were sequenced to exclude the possibility that the variant was a polymorphism in the KRT6A gene (GenBank accession number: NM_005554.3).

Exome capture, sequencing and variant detection

We performed the exome capture using Agilent SureSelect Human All Exon Kits (Agilent, Santa Clara, CA) according to the manufacturer's instructions. Sequencing was performed on a HiSeq 2000 platform with read lengths of 100bp. The mean coverage depth for each sample is 100×. The sequencing reads were described according to NCBI human reference sequence.

SNaPshot confirmation

To confirm the question of somatic mosaicism in the mother, the analysis was performed to quantify the proportion of cells carrying the KRT6A mutation by using SNaPshot (ABI Prism SNaPShot multiplex kit; Applied Biosystems) on an ABI PRISM 3730 genetic analyser according to the manufacturer's instructions. The proportion of normal and mutant DNA was quantified using GeneMapper software

(v4.0; Applied Biosystems). To get mutation ratios of 50%, 25%, 12.5%, 6.25%, 3.13%, and 1.56%, a genomic DNA sample of a heterozygous proband was serially diluted with a sample of a wild-type family member. All experiments were repeated three times.

HiSeq deep sequencing

Besides, we also performed HiSeq deep sequencing. Firstly, We dilute the DNA from the patient's blood with the DNA from the normal by 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128 based on gradient dilution method. Making a standard curve. Then, We designed primer detection mutation site KRT6A (NM_005554.3) c.1385T>A; p.Ile462Asn. (F：TTCCTCTTCCAGTGCGCCAA; R：AGCTGTTGAAGGAGKT CGTGT) And synthesizing fusion primer. (F1：ACACGACGCTCTTCCGATCTT TCCTCTTCCAGTGCGCCAA; R1：TTCCTTGGCACCCGAGAATTCCAAGCTG TTGAAGGAGKTCGTGT) Next step, We carried out the first round PCR.(3min96°C; 15 cycles of 30 seconds 96°C; 30 seconds 60°C; 30 seconds 72°C. End with 5 min incubation at 72°C; pause at 10°C.) And the second round were carried out after screened and purified. (NNNNNN was used to distinguish between different samples. F2： AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACA CGACGCTCTTCCGATCT; R2-x： CAAGCAGAAGACGGCATACGAGATNNN NNNGTGACTGGAGTTCCTTGGCAC CCGAGAAT) (3min96°C; 10 cycles of 15 seconds 96°C; 30 seconds 60°C; 30 seconds 72°C. End with 5 min incubation at 72°C; pause at 10°C.) In the end, Sequencing the PCR products from last step after purified by Illumina Hiseq. And analyzing the number of T and A at the site to be tested in the total read length of each sample.

Results

Patient samples

The proband of this family is a 5-year-old boy from Zhejiang province in China. He developed thickened nails and oral leukokeratosis at birth (Fig. 1a-c), and began developing palmoplantar keratoderma at 2 years old. His sister has similar clinical manifestation characterized with nail discoloration and thickening (Fig. 1d). No abnormalities in the teeth and eyes were noted in the two affected children. There are no unaffected siblings and the phenotypic features of PC were not found in any other family members including their parents.

Identification of a KRT6A mutation by Sanger sequencing and whole exome sequencing

The entire coding and flanking intronic sequences of KRT6A, KRT6B, KRT6C, KRT16 and KRT17 genes were screened for mutations in the two affected children and unaffected parents. A previously reported heterozygous mutation, p.Ile462Asn, was identified in KRT6A in the proband and his sister (Fig. 2a-b). This change was not detected in 100 unrelated, healthy Chinese control individuals (200 alleles). Sequence analysis of the four other keratin genes failed to detect sequence variants in either affected or unaffected individuals of the family (Fig. 2c-d). This mutation was not identified in the parents, in DNA derived from peripheral blood, hair bulbs or buccal smears (Fig. 2e-h). The sperm cells from proband's father was also wildtype (Fig. 2i). Since the two affected children harbored the same pathogenic

mutation, we postulated that one of the parents was mosaic for this variant WES was performed on the two affected children and their parents. Approximately 5 billion bases were sequenced with coverage of 100×. Consequently, the variant was detected in one sequencing read from 86 sequencing reads from DNA derived from the mother's blood (Fig. 3a). The mutation was not identified in DNA derived from the father's blood by whole exome sequencing. The frequency of reads was 47% and 49% in proband and his sister, respectively. The results indicated that the mutation in KRT6A may be from maternal mosaicism in this family.

SNaPshot confirmed the mosaic KRT6A mutation

SNaPshot analysis revealed substantial mutation-level variation in the two affected children and their parents. SNaPshot sequencing revealed mosaicism at level of 2.5% and 4.7% in DNA from the mother's blood and hair bulbs (Fig. 3b-d). No mosaicism was identified in DNA from buccal smears from mother. A non-mosaic wild-type state was found in the healthy father (Data not shown). The frequencies are almost 50% in two affected children (Fig. 3e-f).

HiSeq deep sequencing demonstrated low-grade mosaicism in the mother

We sequencing the DNA sample from patient's younger sister and parents in the same method. Calculating by Y = 1.0007x-0.0036 as Table 1.

Table 1
the number of T and A at the site to be tested in the total read length of patient's family members. HiSeq deep sequencing demonstrated low-grade mosaicism in the patient's younger sister and parents.

Sample	Ref	Alt	ref	alt	total	Alt Detection ratio(X)	Alt Calculating ratio(Y)
DNA from patient's younger sister	T	A	99254	98266	199990	49.14%	48.8099%
DNA from blood of patient's father	T	A	197737	226	199986	0.11%	-0.2469%
DNA from blood of patient's mother	T	A	192109	5917	199992	2.96%	2.6007%
DNA from mouth mucosa of patient's father	T	A	141449	162	143068	0.11%	-0.2467%
DNA from mouth mucosa of patient's mother	T	A	197106	961	199988	0.48%	0.1209%
DNA from hair blubs of patient's father	T	A	13098	47	13265	0.35%	-0.0054%
DNA from hair blubs of patient's mother	T	A	181637	11293	195019	5.79%	5.4348%
DNA from sperm of patient's father	T	A	171327	187	173214	0.11%	-0.2520%

Discussion

To our knowledge, this is the first time that maternal mosaicism has been reported in PC. When we observed two affected children with unaffected parents, one possible explanation was that it was an autosomal recessive disorder. Although there are known recessive cases for other keratin disorders such as epidermolysis bullosa simplex there are no recessive cases of PC with confirmed genetic analysis reported to date. One case previously reported as recessive PC has now been identified as PLACK syndrome with a mutation in the *CAST* gene [7, 10]. Other rarer possibilities to consider in these situations are germ cell mosaicism or paternal identity for accurate genetic counselling. Somatic mosaicism of a mutation in diseases with autosomal-dominant traits gives a few clinical manifestations but is not transmitted to future offspring. Whereas germ cell mosaicism of a mutation in autosomal dominant disorders does not present with a clinical phenotype but the disorder is transmitted to future offspring as observed in the reported cases of PC and neurofibromatosis 1. In some cases, individuals can have a mosaic mutation that affects germline and somatic cells as reported by Shen *et al* [11]. A mother of a dystrophic epidermolysis bullosa patient who presented with a very mild DEB-blistering phenotype was confirmed to be a germline and somatic mosaic. In her skin, 28% of the pro-α1 (VII) procollagen chains contained the mutation, which is higher than the threshold (10–25%) to develop disease, so her clinical manifestation was very mild, with a mild blistering phenotype [12]. Recently, Li *et al* also reported a case of mosaic ichthyosis with different allele frequency in different tissues [13]. In our family, we demonstrate low-grade mosaicism in the mother, with a mutational load of 2.5% and 4.7% in her blood and hair bulbs, respectively. She is not affected. Her children harbouring a mutational load of 50% do express a phenotype. Apparently low-grade mosaicism is tolerated by the body, The threshold for developing PC-K6a must therefore be higher than 4.7% mutant *KRT6A*.

To determine the mosaic mutations, we use ultra deep sequencing, SNaPshot sequencing or pyrosequencing [14]. SNaPshot sequencing was the simplest method for confirmation of the frequency of mosaicism in this case. But it is difficult to detect mosaicism at a level of 2% or less [15]. Ultra deep sequencing and pyrosequencing are sensitive, but they are expensive. It is difficult to implement them in the routine diagnostic laboratory [16]. In our case, we confirmed maternal mosaicism down to 3% using 100× whole exome sequencing and SNaPshot sequencing. These findings indicate the ability of whole exome sequencing, coupled with SNaPshot sequencing confirmatory analyses, to detect low-level mosaicism.

In summary, we report a recurrent p.Ile462Asn mutation in *KRT6A* gene in two children with PC with unaffected parents. This is the first reported case of maternal mosaicism in PC. Although very rare, germ cell mosaicism should be considered when providing genetic counselling for unaffected parents of a child with PC. We demonstrated that WES and SNaPshot sequencing can be useful technologies for confirmation of somatic and germinal mosaicism.

Declarations

Conflicts Of Interest

All authors state that no financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work.

Additional Contributions

We thank the patient's parents for granting permission to publish this information.

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Authors' contributions

Li Ming and Xu Tianyi participated in the overall design and revising the manuscript. Li Yue, Ni Cheng and Pan Chaolan investigated the family history and collected the clinical data of the patient. Wang Yumeng and Yan Ming participated in the experimental data analysis. Li Yue and Cao Qiaoyu participated in the experimental operation and drafting the manuscript. Li Ming and Xu Tianyi are corresponding authors of this manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets and code generated during and/or analyzed during the current study are available in https://github.com/wuhanchun/BMC-materials.

Ethics approval and consent to participate

All authors state that they have obtained written consent from the parents/legal guardians for genetic studies.

Consent for publication

Written consent has been obtained from the parents/legal guardians for publication of clinical details, radiological and biological data.

Competing interests

The authors declare that they have no fnancial or other conficts of interest.

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Figures

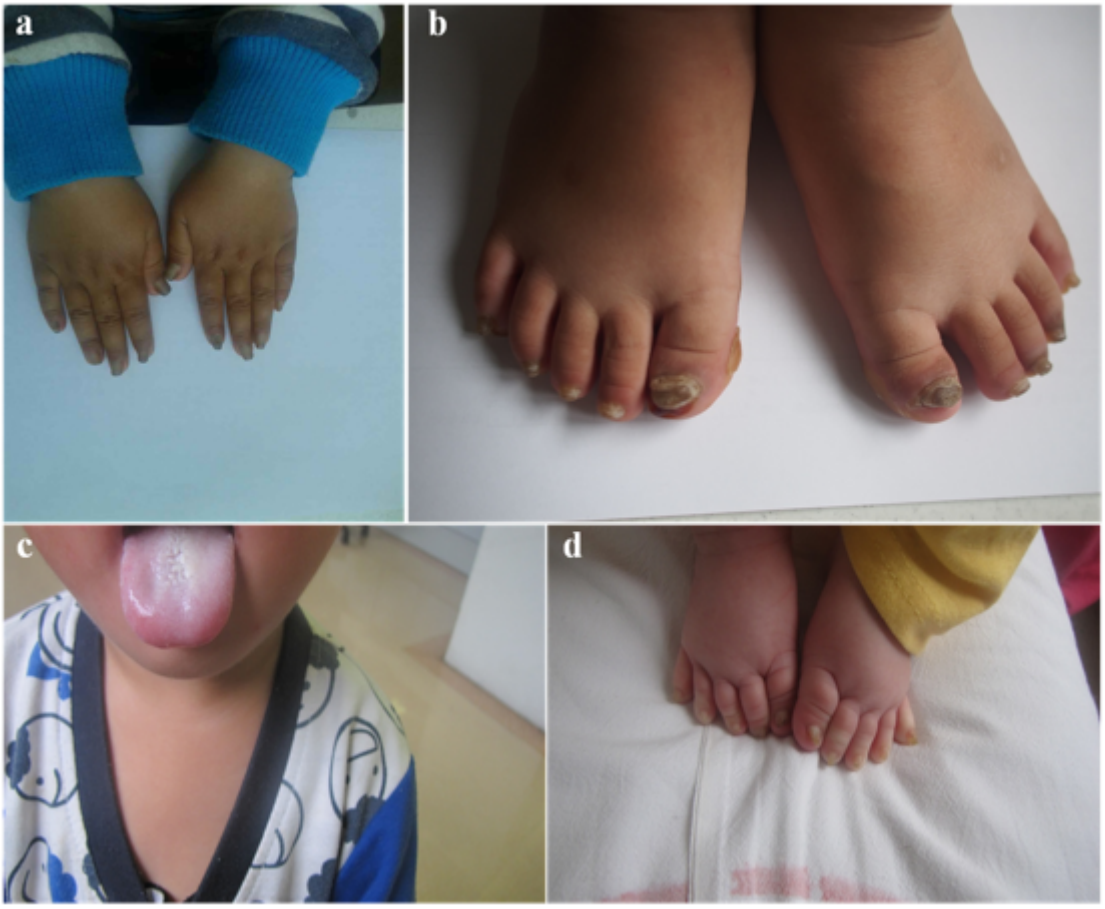

Figure 1

(a-b) Hypertrophic nails of the proband. (c) Oral leucokeratosis of the proband. (d) Hypertrophic nails of the proband's sister.

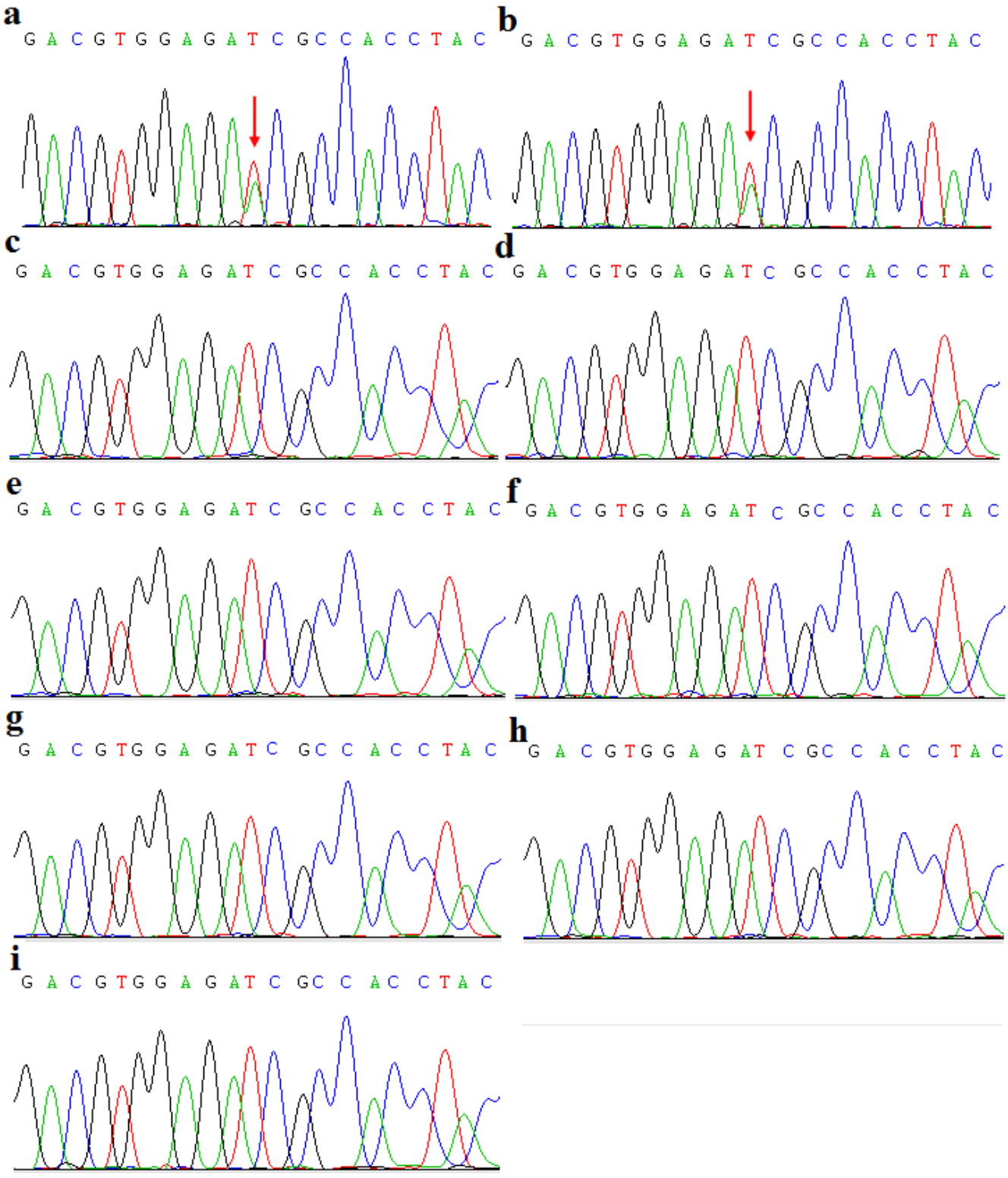

Figure 2

(a) Sequencing analysis revealed a heterozygous c.1385 T>A transition in exon 7 of KRT6A in the proband's genomic DNA from blood. (b) Sequencing analysis revealed a heterozygous c.1385 T>A transition in exon 7 of KRT6A in his sister's DNA from blood. (c-d) The sequence of DNA derived from the parents' blood, (e-f) hair bulbs and (g-h) buccal smears was wild-type. (i) No mutation was identified in father's sperm cells.

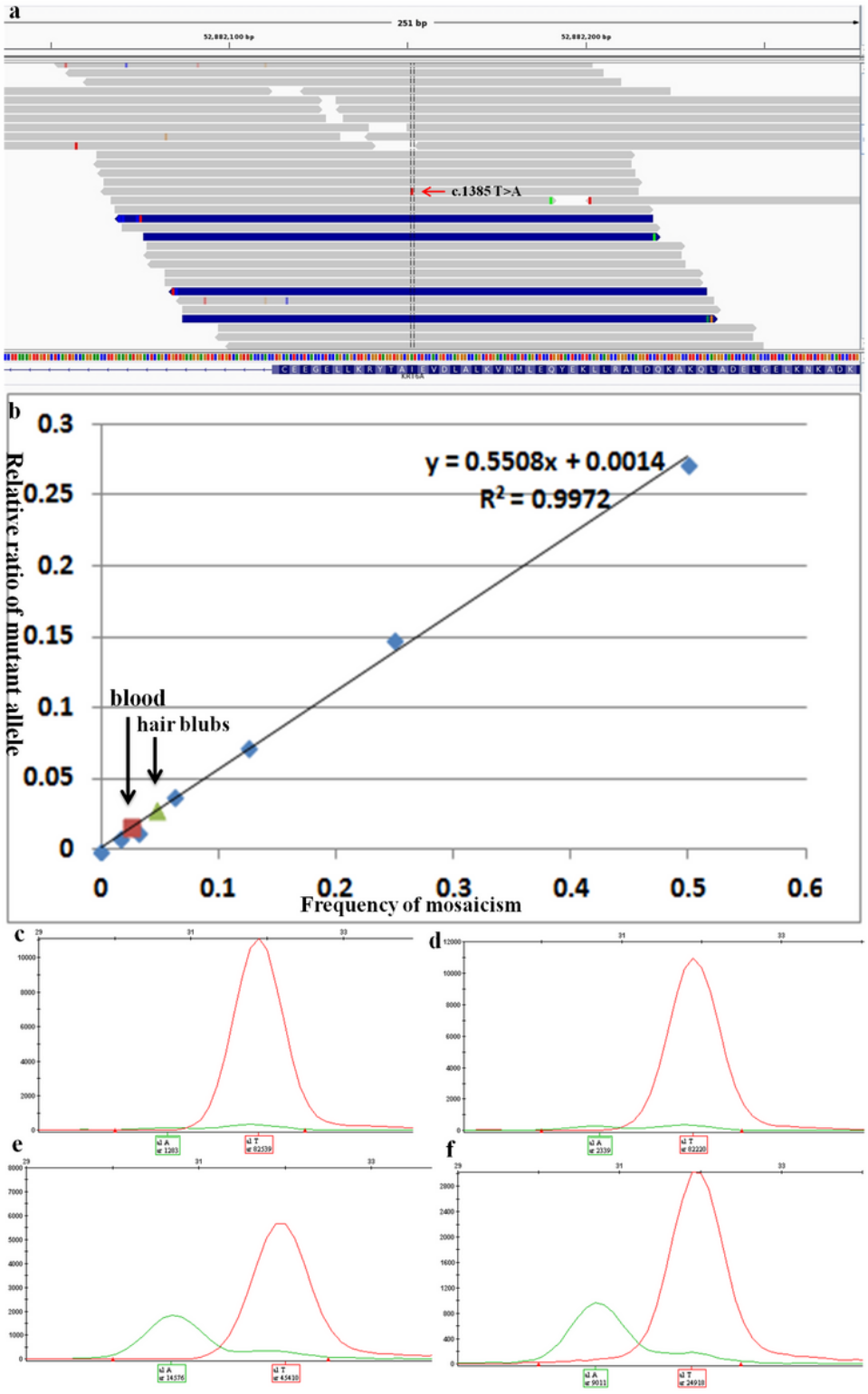

Figure 3

(a) A heterozygous c.1385 T>A transition in exon 7 of KRT6A was identified by WES from mother's blood. The red arrow indicates the variant c.1385 T>A. (b) Standard curve of the mutant allele quantity, derived from serial dilutions of DNA from a heterozygous patient and a normal control, in which 50%, 25%, 12.5%, 6.25%, 3.13%, and 1.56% of the DNA has a mutation. SNaPshot sequencing revealed mosaicism at level of 2.5% and 4.7% in the mother's DNA from blood and hair bulbs. (c) SNaPshot sequencing confirmed the

mother’s DNA from blood carried 2.5% mutant (A). (d) SNaPshot sequencing confirmed mother’s hair bulbs carried 4.7% mutant (A). (e-f) The frequencies of mutant allele were almost 50% in two affected children. The green & red peaks represent the area of T allele and the red peaks represent the area of T allele.