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RESEARCH

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# A cost-effective method for detecting mutations in the human *FAM111B* gene associated with POIKTMP syndrome

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## Abstract

**Background:** Mutations of the human *FAM111B* gene are associated with hereditary fibrosing poikiloderma with tendon contracture, myopathy, and pulmonary fibrosis (POIKTMP), a rare and autosomal dominant multi-systemic fibrosing disease. To date, a total of 36 cases are documented, with eleven associated mutations identified and confirmed by Whole-Exome Sequencing and Sanger sequencing. However, these methods require a certain level of expertise. The *FAM111B* gene was annotated using the SNAPGENE tool to identify various restriction enzymes. The enzymes that cut at the positions where mutations of interest have been reported were selected. The method was implemented using the DNA samples extracted from the skin fibroblast collected from an affected South African family and unrelated control.

**Results:** The findings showed that of the eleven *FAM111B* mutational sites investigated with this method, ten mutations can be identified including the known mutation *FAM111B* NM_198947.4: c.1861T>G (pTyr621Asp) associated with the POIKTMP in South Africa.

**Conclusions:** Limited access to molecular diagnosis contributes to why POIKTMP is rarely diagnosed. Our study describes an inexpensive PCR–RFLP method to screen for POIKTMP *FAM111B* gene mutations. The PCR–RFLP can be used as a cost-effective method for diagnosing *FAM111B* mutations in POIKTMP, and it does not require having robust experience in molecular biology.

**Keywords:** Poikiloderma, PCR–RFLP, Mutations, Molecular diagnosis, Rare disorder

## Introduction

Mutations in the human *family with sequence similarity 111- member B* (*FAM111B*) gene are implicated in a rare and autosomal dominant fibrosing disease, POIKTMP [1–3]. A hereditary multi-systemic fibrosing disease, POIKTMP, is characterized by poikiloderma, tendon contracture, myopathy, and pulmonary fibrosis (hence the disease's name). This disease, first described in a South African family of European descent [2], later identified and confirmed three missense mutations in this family and four other French families of either European or Middle Eastern background by Whole Exome Sequencing (WES) and validated by Sanger sequencing (SG), respectively [3]. To date, there are eleven *FAM111B* de novo or inherited mutations (nine missense, one deletion, and one frameshift) reported in 36 cases. Studies confirmed that *FAM111B* harbors different times of variants that warrant further investigations (Table 1). The cases have emanated from predominantly European, Middle Eastern, and Asian descent, with recently only one case of an American of African ancestry [1, 4–11]. Furthermore, only four cases are from South Africa, a developing country, in the previously described family. The use of WES, SG and other next-generation

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**Table 1** Total classified variants in *FAM111B* gene (UniProt, ClinVar, VarSome & PubMed)

| Coding impact | Pathogenic (%) | Likely pathogenic (%) | Uncertain significance (%) | Benign (%) | Total |
|---|---|---|---|---|---|
| Missense | 60 | 13.3 | 6.7 | 20 | 15 |
| Frameshift | 0 | 33.3 | 33.3 | 33.3 | 3 |
| Inframe indel | 100 | 0 | 0 | 0 | 1 |
| Total | 52.6 | 15.8 | 10.5 | 21.1 | |

sequencing (NGS) technologies has undoubtedly led to the massive generation of previously unknown human DNA sequences and disease diagnosis. However, the accessibility to these technologies in developing countries is limited, which may explain why very few to no reported cases in these countries. Thus, an inexpensive, simple, and rapid genotyping method will be helpful in the validation or screening of families or patients suspected of these disease-causing mutations, especially in families that advanced genomic technologies like WES have already identified a proband.

Polymerase chain reaction coupled with restriction fragment length polymorphism (PCR–RFLP) analysis is a cost-effective screening and genotyping tool in this context. PCR–RFLP involves amplifying a specific DNA sequence target of interest in a gene using polymerase chain reaction (PCR). The PCR–RFLP is also known as cleaved amplified polymorphic sequence. A PCR amplicon is treated by a certain restriction enzyme that cuts the DNA in a unique restriction site, which is known as the recognition site, to generate several DNA fragments in various sizes. Subsequently, the digested amplicons are loaded onto a gel, and an electric field is applied. The differently sized bands will move at varying distances across the gel. The PCR amplified product's restriction digest occurs with a restriction enzyme(s) that distinguishes the wildtype (WT) genotype from the mutated form or vice versa. Furthermore, the selection of genotyping restriction enzymes requires a bioinformatics study of the DNA sequences of mutated (i.e., patients) and WT (i.e., controls) for the gain or loss of restriction sites created by gene mutations. This technique is thus useful for genotyping in the context of single nucleotide polymorphisms (SNPs) confirmation and disease predisposition, diagnostics, and pharmacogenomics [12–14]. Thus, this study described a practical and straightforward PCR–RFLP for screening for the presence of the POIKTMP-associated *FAM111B* mutations and demonstrated this method's use in screening and genotyping the heterozygous NM_198947.4: c.1861T>G (p. Tyr621Asp) *FAM111B* mutation of the South Africa family affected by the POIKTMP disease.

## Materials and methods

### Ethical approval and biospecimen collections

This study received ethical clearance and approval from the Human Research Ethics Committee of the Faculty of Health Sciences, University of Cape Town (HREC REF 577/2020). We had access to previously isolated skin fibroblasts from three members (two affected and one unaffected mother) of the POIKTMP-affected South African family previously described [2, 3]. The patients were clinically diagnosed. The condition manifested with skin changes, which involve the face, including facial telangiectasia, mottled hypo- and hyperpigmentation, papules, and epidermal atrophy. The scalp, facial and body hair are fine and scanty. The tendon contractures lead to progressive digital flexion deformities and abnormalities of the ankles and feet, with disturbance of gait. Pulmonary involvement manifests as progressive dyspnoea in the patients. We also received fibroblasts from an unaffected and unrelated volunteer, which served as a control with the unaffected mother. The cells banked at − 196 °C were thawed and cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal calf serum (FCS) (Thermo Fisher Scientific) at 37 °C with 5% $CO_2$ till confluency.

### Primer design

The human *FAM111B* gene is located on chromosome 11q12.1 [NCBI accession code NM_198947.4] was analyzed and annotated using the SnapGene DNA analysis and visualization software tool version 5.1.7 (GSL Biotech LLC, USA). The software identified identify restriction enzymes that can cut the gene at various positions and allow fragment analysis. We also designed PCR amplification primers flanking the exon 4 region of the gene with the aim to genotype the previously identified *FAM111B* mutation in the South African affected by the POIKTMP disease.

Primers details.

Forward primer: ATGCTATTAATCTGGATGTCC AAAAGGAGG

Reverse primer: CTAACATTCCATGGGTTCAAT CTGATGATC

The *In silico* restriction digests of the amplified WT and mutated *FAM111B* regions and fragment analyses on a 2.5 or 3% agarose gel was also investigated with this software.

### PCR–RFLP molecular diagnosis

Genomic DNA was extracted from the skin fibroblasts using the Omega Biotek EZNA DNA/RNA extraction kit (USA) based on the 'manufacturer's protocol. *FAM111B* targeting primers was synthesized by Inqaba Biotech (South Africa) and used to amplify a 1085 bp region of this gene using the Platinum™ Direct PCR Universal Master Mix kit (ThermoFisher Scientific, USA) following the exact protocol of the PCR kit. The PCR product was cleaned with a PCR cleanup kit (ThermoFisher Scientific, USA) and digested using the NEB High-Fidelity restriction enzyme, BstZ171-HF, for 15 min at 37 °C. The resultant DNA digests were resolved on a 2.5% agarose gel containing 0.005% SYBR™ safe gel stain (Invitrogen, USA) for 80 min at 100 V in Tris–borate-EDTA (TBE) buffer. We analysed and simulated the DNA sequences of the WT and ' 'patient's mutations in *FAM111B* gene, respectively, to identify restriction enzymes that will be useful for genotyping. A total of 10 mutations [NM_198947.4: c.1247T>C (p.Phe416Ser), c.1261_1263delAAG (p.Lys421del), c.1289A>C (p.Gln430Pro), c.1462delT (p.Cys488Valfs*21), c.1861T>G (p.Tyr621Asp), c.A1873C (p.Thr625Pro), c.1874C>A (p.Thr625Asn), c.1879A>G (p.Arg627Gly), c.1883G>A (p.Ser628Asn) and c.1884T>A (p.Ser628Arg)], were analysed with the identified restriction enzymes and the predicted DNA fragments' length produced by these enzymes are listed in Table 2. Since the mutations in the *FAM111B* have been principally associated with the POIKTMP, then we performed an *insilico* genetic interactions using the GENEMANIA tool https://genemania.org for the establishment of plausible biological functions/interactions with *FAM111B* protein, likely to explain the biological complexity of the disease.

## Results

### PCR–RFLP genotyping

The c.1861T>G (p. Tyr621Asp) previously described in the South African family was confirmed in this study with this method (Fig. 1). The WT was expected to be digested by the restriction enzyme to produce DNA fragments with sizes of 740 and 345 bp. The heterozygous missense mutation gave three DNA fragments with sizes 1085, 740, and 345 bp in the affected siblings, while the unaffected mother and unrelated control had the DNA band pattern for the WT genotype (Fig. 1).

Furthermore, we confirmed the PCR–RFLP genotyping method to be able to detect ten of the reported mutations; however, the position of the eleventh *FAM111B* gene mutation c1881 C>T (p. Arg672Ser) was not verified in the study (Table 2). The loss of the *BstZ17I* restriction site in the mutated form of this gene at this position signify lack of cleavage in the DNA fragment.

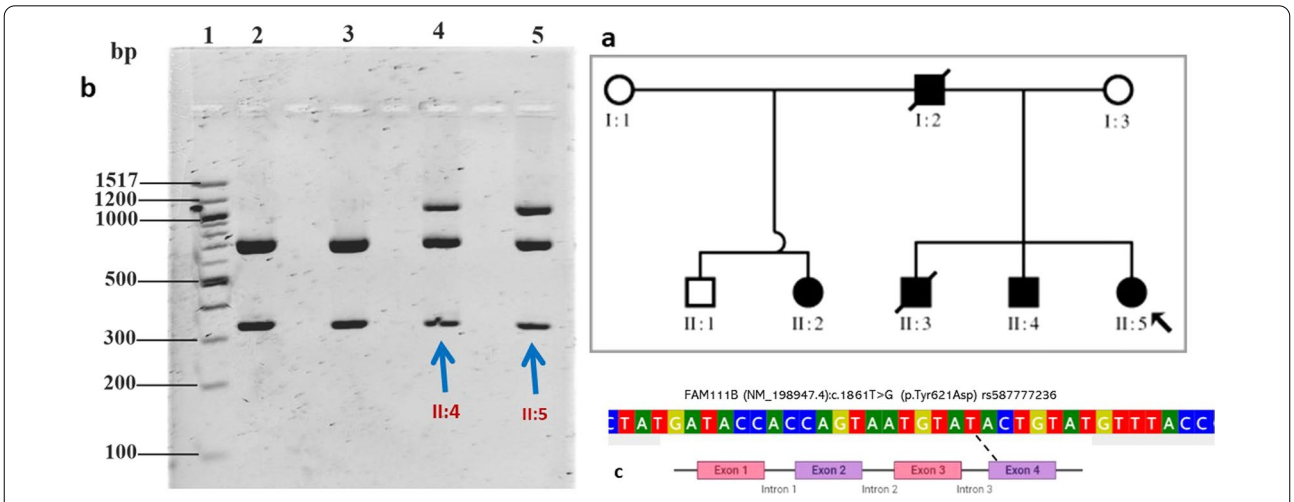


**Fig. 1** **a** PCR–RFLP genotyping of the South Africa family with the FAM111B heterozygous [NM_198947.4: c.1861T>G (p.Tyr621Asp)] gene mutation at exon 4. Lanes 1: 100 bp DNA ladder (NEB, USA), 2: Familiar control (WT i.e., unaffected mother), 3: unrelated control (WT), 4: POIKTMP (mutant) affected family member, 5: a second POIKTMP affected family member. The expected band sizes: WT (i.e., T/T genotype) = 740 and 345 bp, and heterozygous mutant (i.e., T/G) = 1085, 740 and 345 bp. **b** Pedigree analysis of the family. **c** The description of the location of the mutation of interest reported in the patients

**Table 2** *FAM111B* gene mutations, genotyping restriction enzymes and DNA fragment lengths

| No | *FAM111B* gene mutation | Type of mutation | Restriction enzymes | DNA fragment lengths (bp) |
|---|---|---|---|---|
| 1 | c.1247T>C (p. Phe416Ser) (12) | Missense | AvaI/BsoBI/Eco88I/XmnI | T/T = 1085 bp (i.e., Noncutter)<br>T/C = 1085, 958, and 127 bp<br>C/C = 958 and 127 bp |
| 2 | c.1261_1263delAAG (p. Lys421del) (5) | Deletion | Hin4I | A/A = 397, 243, 215 and 3 × 32[#] bp<br>A/G = 560, 390, 243, 215 and 3 × 32[#] bp<br>G/G = 560, 243, 275 and 2 × 32[#] bp |
| 3 | c.1289A>C (p. Gln430Pro) (3, 4) | Missense | TasI/MluCI | A/A = 251, 194, 161[†], 146[†] and 79 bp<br>A/C = 251, 194, 161[†], 146[†], 120[†] and 79 bp<br>C/C = 251, 194, 161[†] and 146[†] bp |
| 4 | c.1462delT (p. Cys488Valfs*21) (16) | Frameshift | Hpy991/HpyCH4III | T/T = 1085 bp (i.e., Noncutter)<br>delT/T = 1085, 739, and 345 bp<br>C/C = 739 and 345 bp |
| 5 | c.1861T>G (p. Tyr621Asp) (2, 7) | Missense | BstZ17I/Bst 11071/BssNAI | T/T = 740 and 345 bp<br>T/G = 1085, 740 and 345 bp<br>G/G = 1085 bp (i.e., Noncutter) |
| 6 | c.1873A>C (p. Thr625Pro) (13) | Missense | Hpy8I | A/A = 395, 345, 284, 50 and 11* bp<br>A/C = 395, 345, 295[†], 284[†], 50 and 11* bp<br>A/A = 395, 345, 295, 50 bp |
| 7 | c.1874C>A (p. Thr625Asn) (4) | Missense | MseI/TruI/Tru9I/SaqAI | C/C = 393 and 238 bp[#]<br>C/A = 393, 238, 145 bp[#]<br>A/A = 393 and 145 bp[#] |
| 8 | c.1879A>G (p. Arg627Gly) (7) | Missense | StyI/ BsaJI | A/A = 592, 480 and 13* bp<br>A/G = 592, 480, 317, 275 and 13* bp<br>G/G = 480, 317, 275 and 13* bp |
| 9 | c.1883G>A (p. Ser628Asn) (7) (4, 10) | Missense | ApoI/XapI | G/G = 715, 161, 120 and 89 bp<br>G/A = 715, 552, 163[$], 161[$], 120 and 89 bp<br>A/A = 552, 163[$], 161[$], 120 and 89 bp |
| 10 | c1881 C>T (p. Arg672Ser) (14)<br>This reported mutation was unverifiable | Missense | – | – |
| 11 | c.1884T>A (p. Ser628Arg) (1) | Missense | BsaBI/BseJI | T/T = 1085 bp (i.e., Noncutter)<br>T/A = 1085, 767 and 318 bp<br>A/A = 767 and 318 bp |

[*] Band might be too small to visualize

[#] Bands < 100 bp (i.e., between 7 and 92 bp) will be created by these enzymes and is advisable to run these bands off the gel for proper visualization of the > 100 bp band that produces the distinctive band patterns required for genotyping

[$] A thick band comprising the 163 and 161 bp may be observed on the agarose gel (we recommend a 3.0% agarose gel to resolve the DNA fragments)

[†] These DNA fragments genotypes may prove difficult to separate on a 2.5% agarose gel. We recommend a 3–4% agarose gel or 12–15% polyacrylamide gels (using 20 bp DNA ladder) and running the electrophoresis at a voltage of 35–40 V under cold conditions to separate these bands

N.B. Values in parenthesis are the references describing these mutations

Also, the mutations that the predicted restriction enzyme would digest the DNA fragments < 20 bp (e.g., c.1261_1263delAAG (p. Lys421del and c.1873A>C (p. Thr625Pro) was difficult to visualise on a 2.5% gel. However, running the DNA fragment with this size off the gel and relying on the fragments > 20 bp containing the genotyping DNA bands should worked. Moreover, the DNA fragments produced half size differences of 10–20 bp [e.g., c.1289A>C (p. Gln430Pro) and c.1873A>C (p. Thr625Pro)] and may be difficult to resolve on a 2.5% agarose gel as well.

The results of the insilico genetic interaction analyses indicated that the human FAM11B is a crucial gene involved in various physiological functions. The physical interactions with other neighboring genes were estimated at 77.64%, co-expression 8.01%, and predicted, co-localization, genetic interactions, pathway, shared protein domains amounted to approximately 15%, with biological functions linked to kinetochore, chromatin remodeling at centromere, CENP-A containing chromatin organization condensed chromosome, centromeric region nucleosome organization histone exchange centromere complex assembly (Fig. 2).

## Discussions

*FAM111B* represents an uncharacterized protease involved in DNA repair, cell cycle regulation, and apoptosis. This gene encodes a protein with a trypsin-like cysteine/serine peptidase. Its involvement in kinetochores functions as identified in the insilico analyses in

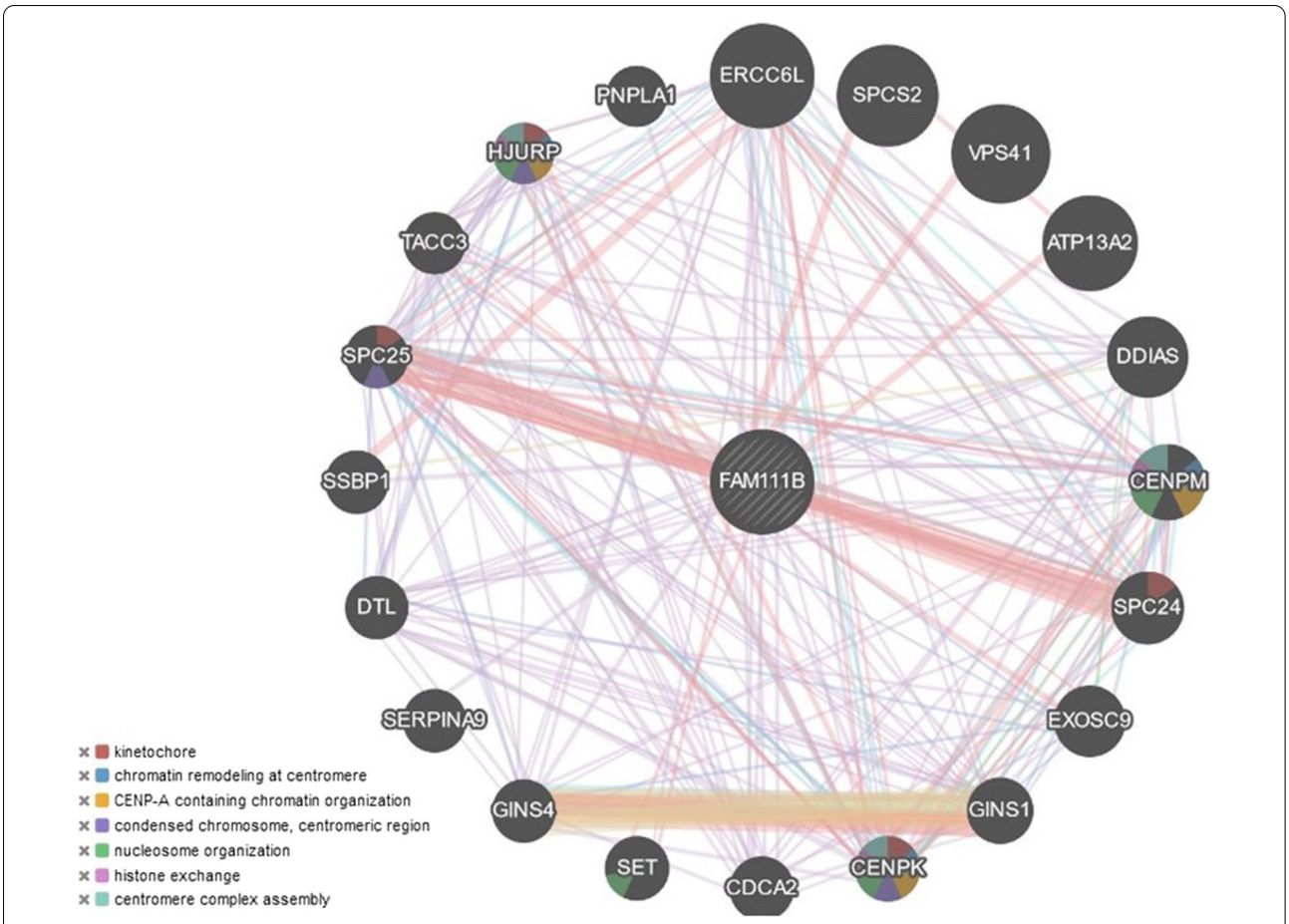


**Fig. 2** Gene–gene network analyses performed by genemania tool highlighting the interactions of *FAM111B* protein with proteins sharing functional interactions

this study, described why *FAM111B* remains a major gene associated with multiple disorders such as the POIKTMP. The kinetochores are large protein assemblies that connect chromosomes to microtubules of the mitotic and meiotic spindles to distribute the replicated genome from a mother cell to its daughters. The kinetochore function for overall body growth and provide new insight into the cellular mechanisms.

Our study demonstrated the use of the PCR–RFLP method in genotyping mutations in the *FAM111B* gene and performed gene enrichment analyses. We validated the South African *FAM111B* heterozygous [NM_198947.4: c.1861T>G (p. Tyr621Asp)] gene mutation previously identified by our group. There are over twenty methods for SNP genotyping. The PCR–RFLP is a rapid and sensitive method for the detection of gene polymorphism. The concept of PCR–RFLP is based on the presence or absence of a particular recognition site in the target sequence, which usually does not exceed eight nucleotides in length. It is highly valuable in genotyping, including wide applications in agricultural, medical, microbiology and genomic medicine.

Although the use of WES, NGS and Sanger sequencing remains the preferred choice for screening and validation of disease-causing or predisposing mutations and SNPs, it is a stark reality that this sequencing technology remains inaccessible to underprivileged communities. Furthermore, though the use of RFLP in genotyping is not new, we have described for the first time the use of this method in genotyping reported mutations of the human *FAM111B* gene previously obtained by Sanger sequencing. The analysis of the *FAM111B* gene revealed that the reported mutations are in the exonic regions particularly in the exon 4 (Fig. 1). The gene enrichment analyses suggest that mutations in the *FAM111B* gene may affect the normal function of the protein and may have severe consequences as proposed and demonstrated by some recent studies [15, 16].

To tackle the fragments that were difficult to resolve on a 2.5% agarose gel, we recommend running the restriction digested DNA fragments on a 3.5–4% agarose gel or a 12–15% polyacrylamide gel. The electrophoresis, in either case, must be run at a low voltage. Otherwise, the use of specialised electrophoresis buffers with low-molarity conductive properties may be employed [17, 18].

Moreover, this method mostly requires the standard reagents and equipment already present in genetics research or clinical diagnostic laboratories. Although this study used Snapgene (a licensed software) for in silico gene annotation and analysis, there are other free bioinformatics tools readily available to identify genotyping restriction enzymes [19–21]. Furthermore, though we used DNA from skin fibroblasts in this study, other easily accessible DNA sources such as blood and saliva may suffice without the need for an expensive DNA extraction kit. Also, the use of post PCR clean-up kit indicated in this study is not necessary. The limitations to this method are that to conduct a successful PCR–RFLP, a high concentration of amplicons is needed because of the limited capability of agarose gels to separate molecules compared with the highly sensitive polyacrylamide gels. Also, sometimes the reactions must be incubated for 17–24 h if the enzyme failed to cut at 4 h. It is important to apply this method in screening for mutations in the *FAM111B* gene to rapidly determine which causal mutations are present in patients. The limitations to this study can be found in the rarity of the POIKTMP cases. To date, one two families in South Africa have identified with this disease which makes the genetic contributions to this disease unknown. Also, not all the reported mutations were genotyped using the PCR–RFLP method in this study to be able to ascertain its full potential as a genotyping method of choice in low-resource settings.

## Conclusions

We described a simple and cost-effective genotyping method for the genotyping of the reported *FAM111B* gene mutations. We also demonstrated using this method for the in vitro genotyping the POIKTMP affected South African family's heterozygous *FAM111B* mutation. This method will prove useful in screening patients suspected of POIKTMP disease and genotyping large sample sizes in resource constraint clinical laboratories. The *insilico* genetic interactions study showed that there are couple of genes that deserved to be investigated alongside the *FAM111B* gene based on robust co-sharing of biological functions that are point of interest for POIKTMP.

### Abbreviations

*FAM111B*: Family with sequence similarity 111- member B; WES: Whole exome sequencing; SG: Sanger sequencing; NGS: Next-generation sequencing; PCR–RFLP: Polymerase chain reaction coupled with restriction fragment length polymorphism; WT: Wildtype; SNPs: Single nucleotide polymorphisms; TBE: Tris–borate-EDTA.

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### Author contributions

AA conceptualized the study, performed experiments, and wrote the draft manuscript. CR assisted with the experiments and collated the reported *FAM111B* mutations. MM assisted with the study, wrote the abstract and reviewed the manuscript, OGO performed major analyses and revised the manuscript and NK recruited patients and facilitated the samples collections for the study and corrected the manuscript. All authors read and approved the final manuscript.

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### Availability of data and materials

Data used in this study are available by request from the authors.

## Declarations

### Ethics approval and consent to participate

This study received ethical clearance and approval from the Human Research Ethics Committee of the Faculty of Health Sciences, University of Cape Town (HREC REF 577/2020).

### Consent for publication

Study participants approved the use of data for publication without any form of identification.

### Competing interests

The authors have no conflict of interest to declare.

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## Publisher's Note