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CASE SERIES

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Homozygous Mutations in *Thyroid Peroxidase* (*TPO*) in Hypothyroidism with Intellectual Disability, Developmental Delay, and Hearing and Ocular Anomalies in Two Families: Severe Manifestation of Untreated TPO-deficiency Poses a Diagnostic Dilemma

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Intellectual disability (ID) involves compromised intellectual, learning and cognitive skills, and behavioral capabilities with reduced psychomotor skills. One of the preventable causes of ID is congenital hypothyroidism (CH), which may be due to biallelic mutations in *thyroid peroxidase* (*TPO*). In low- and middle-income countries with no newborn screening programs, CH poses a great risk of ID and long-term morbidity. We report two large Pakistani families with a total of 16 patients afflicted with CH. Detailed clinical and behavioral assessments, SNP-based homozygosity mapping, linkage analysis, and exome sequencing were performed. Initially, affected individuals were referred as suffering ID (in 11 of 16 patients) and developmental delay (in 14). Secondary/associated features were verbal apraxia (in 13), goiter (in 12), short stature (in 11), limb hypotonia (in 14), no pubertal onset (five of 10 of age ≥14 years), high myopia (in eight), muscle cramps (in six), and in some, variable microcephaly and enuresis/encopresis, fits, chronic fatigue, and other behavioral symptoms, which are not characteristics of CH. Molecular genetic analyses led to the discovery of homozygous variants in *TPO*: novel missense variant c.719A>G (p.Asp240Gly) in family 1 and rare c.2315A>G (p.Tyr772Cys) in family 2. In low-resource countries where neonatal screening programs do not include a CH test, the burden of neurodevelopmental disorders is likely to be increased due to untreated CH. Secondly, in the background of the high prevalence of recessive disorders due to high parental consanguinity, the severe manifestation of *TPO*-deficiency mimics a wide range of neurological and other presentations posing a diagnostic dilemma.

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Abbreviations: CH, congenital hypothyroidism; ID, Intellectual disability; LOD, logarithm of the odds; MIM, Mendelian inheritance in Man; TDH2A, thyroid dyshormonogenesis 2A; *TPO*, *thyroid peroxidase*, SNP, single nucleotide polymorphism; WES, whole exome sequencing.

Keywords: verbal apraxia, goiter, short stature, high myopia, microcephaly, thyroid dysgenesis

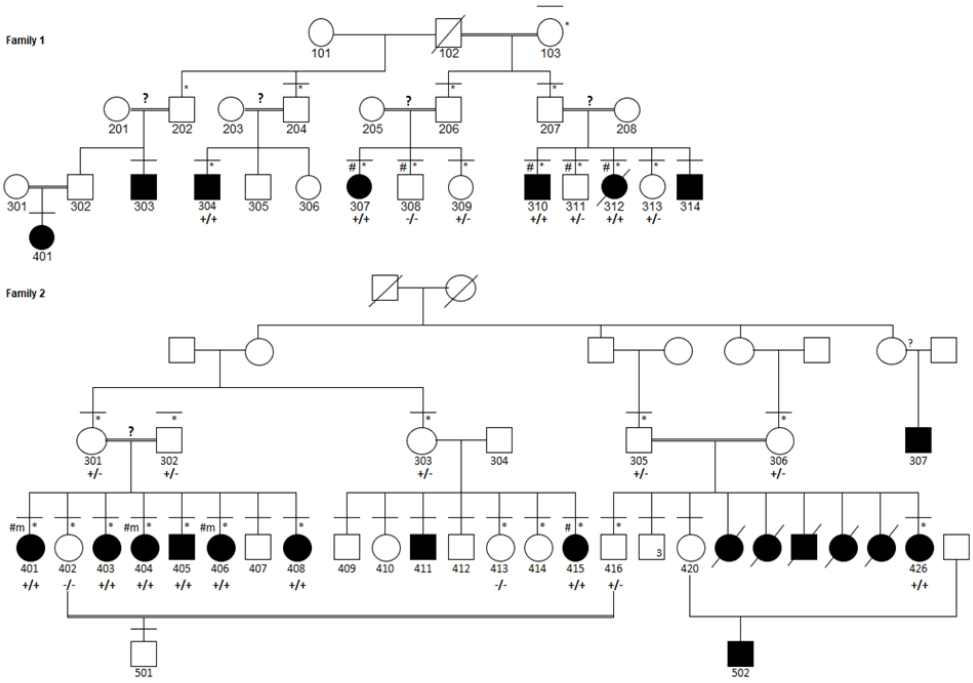

Figure 1. **Pedigrees**. Genotypes for *TPO* c.719A>G. in family 1 and for *TPO* c.2315A>G in family 2 are presented. +, variant; -, reference allele. DNA was available for individuals marked *. SNP genotype data were generated for individuals marked #. Horizontal line above the symbol indicates that clinical examination was performed. #m, a mixture of DNA samples of three affected sisters (401, 404 and 406 in family 2) was SNP genotyped.

INTRODUCTION

Congenital hypothyroidism (CH; MIM 275200) due to thyroid dyshormonogenesis 2A (TDH2A) is one of the common congenital endocrine disorders in infants with an incidence of 1:3000 to 1:4000 [1,2]. It has characteristic symptoms including jaundice, constipation, large fontanel, swollen tongue, and floppy muscle tone. In many instances, however, partly due to either some residual thyroid function or transplacental passage of maternal thyroid, newborns with CH either do not show any symptoms or exhibit just a mild phenotype such as poor feeding, excessive sleeping, and constipation. Hence, the diagnosis for the disorder is generally not straightforward [1,2]. Symptoms in children may include poor growth, delayed development of permanent teeth, delayed onset of puberty, and poor mental development [2,3]. In low- and middle-income countries, including Pakistan, where there is no newborn screening program, delayed diagnosis of hypothyroidism can lead to the most severe outcomes of CH including intellectual disability (ID) and developmental delay [4,5], thus mimicking other neurodevelopmental disorders. This may be trivial particularly in the societies with high prevalence of inherited disorders due to a high rate of parental consanguinity.

The majority of the genetically characterized cases of hypothyroidism have biallelic variants in *thyroid peroxidase* (*TPO*; MIM 606765) [6], and in the majority of those TDH2A cases the disease manifests with the characteristic symptoms of congenital hypothyroidism such as goiter and total iodide organification defect [7,8]. TPO protein catalyzes key reactions in thyroid hormone synthesis and coupling of some of the hormonally inert iodotyrosine residues (monoiodotyrosine and diiodotyrosine) to form the hormonally active iodothyronines thyroxine (T4) and triiodothyronine (T3) [7,9].

In this study, we aimed to identify the genetic basis of hypothyroidism in two families with an unusual presentation of ID and microcephaly and to compare their clinical and molecular features with previously reported cases of TDH2A. High throughput data analyses methods including whole genome SNP-based genotyping, linkage analyses, homozygosity mapping, whole exome sequencing (WES), and Sanger sequencing were employed. Studies on the families were initiated because of the primary presentation of ID and microcephaly and the diagnosis of hypothyroidism was reached very late. To date, ID has been described in four TDH2A families only [5,10,11], with somewhat different features than our patients.

MATERIALS AND METHODS

Family and Clinical Investigations

Peripheral blood samples were collected after obtaining informed consent according to the Helsinki II declaration. The study protocol was approved by the ethical review committee of Quaid-i-Azam University (DAS-1070) and the Istanbul Technical University Ethics Review Board (MBG.22/2014).

Family 1 originates from a rural area of Punjab, Pakistan. The disease was seen in five branches over two generations, and almost all marriages have known consanguinity (Figure 1A). A detailed pedigree was constructed, and clinical and pathological data of all seven affected subjects were collected. Eight unaffected relatives were also examined. Thyroid hormone levels were investigated in five patients, thyroid scan was performed in one and thyroid ultrasonographic evaluations in four. Photographs of all patients and X-ray films of three patients were obtained. Blood samples of eight participants including four patients were available for the genetic study.

Family 2 originates from a rural area of Northern Pakistan. The disease was seen in four branches over three generations (Figure 1B). Seventeen affected members were reported, five of whom were deceased. Blood samples were obtained from 16 family members (eight affected, eight unaffected) who were also physically examined with the help of local physicians. The assessment of ID was based on the criteria of American Psychiatric Association (APA, DSM-5 instrument): 1. Deficits in intellectual functioning (ie, reasoning; problem solving; planning; etc.), 2. Deficits or impairments in adaptive functioning (ie, communication, social skills; etc.), and 3. Onset of symptoms during the developmental period [12].

Genetic Analyses

For family 1, genotype data for 710K SNP markers for three affected (307, 310, and 312) and two unaffected (308 and 311) members were generated using Illumina Human OmniExpress-24 BeadChip (Figure 1A). Multipoint linkage analysis was performed using Allegro in easyLINKAGE v.5.08, assuming autosomal recessive inheritance with full penetrance and a disease allele frequency of 0.001 [13]. In order to not miss any shared homozygosity region, HomozygosityMapper was applied.

In family 2, a mixture of DNA samples of three affected sisters (401, 404, and 406) and separately of a sample of affected cousin 415 were SNP genotyped, similarly to family 1 (Figure 1B). Intervals >1Mb with shared homozygosity were detected with HomozygosityMapper.

Exome sequencing was performed for affected individuals 310 in family 1 and 403 in family 2 using the Agilent SureSelect Target Enrichment System and the Illumina HiSeq2000 platform. The reads were mapped to the reference genome using Burrows-Wheeler alignment (BWA-0.5.9). Variant calling was performed with Sequence Alignment/Map tools (SAMtools-0.1.14) and Annotate Variation (ANNOVAR) to annotate variants (as per [13,14]). In candidate regions, homozygous rare (frequency <0.01) and novel variants were selected according to the information in public databases gnomAD, 1000-Genome, and ESP6500, as described previously [13,14]. Sanger sequencing was carried out to validate each causative variant and its segregation in the respective family.

RESULTS

Clinical Findings: Family 1

According to family elders all patients exhibited variable features such as lethargy, chronic fatigue, excessive sleeping, recurrent fever, goiter, jaundice, constipation, and hypotonia (Table 1). In the early childhood, they had delayed developmental milestones, including crawling, walking, speech, and reduced cognitive skills. However, the diagnosis of hypothyroidism was delayed due to inadequate medical facilities in their remote rural areas.

Upon a recent clinical examination, we found that of the four patients with reduced motor and cognitive skills, two (307 and 312) had ID which was assessed as severe to profound as per criteria of the APA [12], reduced motor and cognitive skills, developmental delay, verbal apraxia, short stature, protruding tongue, physical disability with inability to stand up or walk without support, no pubertal onset, enuresis, and encopresis (Table 1). Two others (310 and 314) were slow learners and had strabismus. Patients did not have ataxia. They exhibited various behavioral problems which were more severe in the two with ID (Table 2). Those two patients exhibited hand banging, tantrums, self-mutilation, bipolar episodes, sensitivity to food and taste, thumb sucking, and poor awareness of clothing. The behavioral anomalies observed in the majority of patients were aggression, hyperactivity, and attention deficit.

Recent hormonal investigations revealed low levels of T4 and very high levels of TSH (Appendix A: Supplemental Table 1). Ultrasonic investigation of thyroid glands depicted spongy appearance with hypertrophy and cyst formation (Supplemental Table 2). Radiological examination of upper limbs demonstrated poorly calcified and atrophic epiphyses and hypoplastic elbow and wrist joints, indicative of delayed maturation (Supplemental Table 3; Supplemental Figure 1). Patients started thyroxin therapy but had poor compliance and had recurrent thyroxin withdrawal symptoms.

Table 1. Physical and Clinical Features of Members with Hypothyroidism in Family 1

Pedigree IDs	303	304	307	310	312	314	401	
Sex, age (years)	M, 19	M, 23	F, 15	M, 12	F, 14	M, 9	F, 7	Concordance
Clinical features								
Intellectual disability	−	−	severe	− (slow learner)	severe	− (slow learner)	−	2/7
Goiter	+	+	+	+	+	+	−	6/7
Reduced motor and cognitive skills	−	−	+	+	+	+	−	4/7
Verbal apraxia	−	−	+	−	+	+	+	4/7
Short stature	−	−	+	−	+	−	−	2/7
Strabismus	−	−	−	+	−	+	−	2/7
Hypotonia (limbs)	−	+	+	+	+	+	−	5/7
Physical disability#	−	−	+	−	+	−	−	2/7
Protruding tongue	−	−	+	−	+	−	−	2/7
No pubertal onset	−	−	+	NA	+	NA	NA	2/7
Enuresis and encopresis	−	−	+	−	+	−	−	2/7
Chronic fatigue	+	+	+	+	+	+	−	6/7
Other	−	−	childish face	−	−	−	abdominal swelling	
Developmental features								
Developmental delay	−	−	+	+	+	+	+	5/7
Crawling and walking late	−	−	+	−	+	+	−	3/7
Speech delay	+	−	+; poor speech	+	+; poor speech	+	+	6/7
Hearing late	−	−	+	−	+	−	−	2/7

#unable to stand or walk without help; +, feature present; −, feature absent; NA, not applicable

Table 2. Behavioral Features in Patients in Family 1

Pedigree ID	303	304	307	310	312	314	401	
Sex, age (years)	M, 19	M, 23	F, 15	M, 12	F, 14	M, 9	F, 7	Concordance
Aggression, short tempered	+	+	+	+	+	+	+	7/7
Attention deficit	−	−	+	+	+	+	+	5/7
Hypoactive/lethargy	−	−	+	−	+	+	+	4/7
Bipolar episodes	−	−	+	−	+	−	−	2/7
Head banging, tantrums, self-mutilation	−	−	+	−	+	−	−	2/7
Sensitive to food and taste	−	−	+	−	+	−	−	2/7
Sleep disturbance	−	−	+	−	+	−	−	2/7
No awareness of clothes	−	−	+	−	+	−	−	2/7
Thumb sucking	−	−	+	−	+	−	−	2/7
Unable to attend school	−	−	+	+	+	+	−	4/7
Self-care (absent)	−	−	+	−	+	+	−	3/7
Using Thyroxin tablet since (age years)	1.5	2 months	2	1.5	2	1.5	1.5	7/7
Thyroxin withdrawal symptoms present	+	+	+	+	+	+	+	7/7

+, feature present; −, feature absent

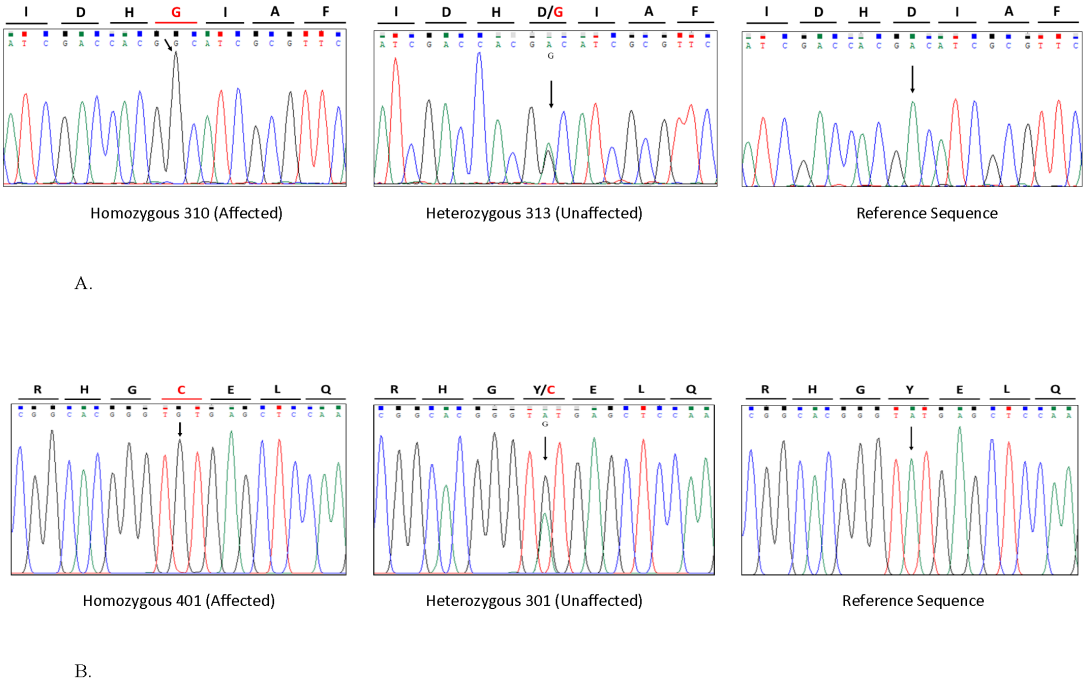

Figure 2. **Electropherograms showing causative *TPO* variants, c.719A>G (p.Asp240Gly) in family 1 (A) and c.2315A>G (p.Tyr772Cys) in family 2 (B)**.

Clinical Findings: Family 2

All patients had ID, reduced motor and cognitive skills, short stature, developmental delay, verbal apraxia, chronic fatigue, cold intolerance, and behavioral problems (Tables 3,4). Patients also had limb hypotonia, more pronounced in lower limbs, and complained of muscle cramps. Eight patients had high myopia and two patients had strabismus. Some of the patients had other variable features such as frequent faints, infantile epileptic episodes, delayed wound healing, hair loss, sun avoiding behavior, and tooth agenesis (Table 3). Anthropometric measurements showed delayed milestones, short stature (<1 or 5 percentile), and low weight. Head circumference (OFC) was <5 percentile in three of the patients (Supplemental Table 4). A recent hormonal profiling in two patients revealed low T3 and T4 and elevated TSH (Supplemental Table 1).

Reportedly all patients had intra-uterine growth retardation. They had small heads at birth and were diagnosed as microcephalic. In the postnatal period, they had delayed developmental milestones, however, later the heads attained normal size. In the recent evaluation, only the youngest affected female of age 6 (415) was observed to have microcephaly while all others had normal OFC. Patients 411 and 426 had seizures in early infancy. Pubertal onset was delayed in three of the six patients aged 15 years or older. Patients had no formal schooling but were able to do some simple tasks under supervision, undertake self-care, and have a sense of self-respect but have not acquired any occupational skills. Five affected siblings had died shortly after birth due to lung infections and multiple lumps in the neck.

Genetic Findings

In family 1 linkage analysis yielded a maximal LOD score of 3.55 for only one region >1 Mb in size: between rs10195681 (nucleotide 18674) and rs10165836 (3433368) at 2p25.3 (Supplemental Figure 2). We also attempted to investigate whether the ID in individuals 307 and 312 could be due to another genetic defect. In search of such a possible ID locus, linkage analysis was performed under a recessive model using the genotype data of those two patients with ID and of unaffected siblings. We did not find a candidate region.

Variants listed within the candidate region at 2p25.3 in the exome sequence file were investigated. The only candidate variant was homozygous *TPO* c.719A>G (p.Asp240Gly; NM_000547) in exon 7 (Figure 2). The variant is novel and predicted as damaging to the protein by computational algorithms (Supplemental Table 5). It segregated with the disease (Figure 2; Supplemental Figures 3 and 4) and was submitted to ClinVar (VCV000869101.1).

In family 2, SNP HomozygosityMapper and MS Excel analysis led to the detection of four candidate regions

Table 3. Physical and Clinical Features of Patients in Family 2

Pedigree IDs	401	403	404	405	406	408	411	415	426	Concordance
Sex, age (years)	F, 25	F, 21	F, 19	M, 17	F, 15	F, 10	M, 15	F, 6	F, 12	
Clinical features										
Intellectual disability	mild	mild	mild	mild	mild	mild	mild	mild	moderate	9/9
Goiter	++	++	−	+	+	−	++	+	−	6/9
Reduced motor and cognitive skills	+	+	+	+	+	+	+	+	+	9/9
Verbal apraxia	+	+	+	+	+	+	+	+	+	9/9
Short stature	+	+	+	+	+	+	+	+	+	9/9
Strabismus	−	−	−	−	−	−	−	++	++	2/9
High myopia	+	+	+	+	+	+	−	+	+	8/9
Hypotonia (limbs)	+	+	+	+	+	+	+	+	+	9/9
Muscle cramps	+	+	+	+	+	+	−	−	−	6/9
No pubertal onset	−	−	−	+	+	N/A	+	N/A	N/A	3/7
Enuresis/encopresis	−	+	−	−	−	−	−	+	+	3/9
Chronic fatigue	++	++	++	++	++	++	++	++	++	9/9
Cold intolerance	++	++	++	++	++	++	++	++	++	9/9
Frequent faints	−	+	+	−	−	−	−	−	−	2/9
Tooth enamel decay	−	−	+	−	−	−	−	−	+	2/9
Developmental features										
Developmental delay	++	++	++	++	++	++	++	++	++	9/9
Crawling and walking late	++	++	++	++	++	++	−	−	++	7/9
Speech delay	+	+	+	+	+	+	+	+	+	9/9
Hearing late	−	+	−	−	−	−	−	−	+	2/9

+, feature present; ++, severe phenotype; −, feature absent; N/A, not applicable

Table 4. Behavioral Features in Patients in Family 2

Pedigree ID	401	403	404	405	406	408	411	415	426	Concordance
Sex, age (years)	F, 25	F, 21	F, 19	M, 17	F, 15	F, 10	M, 15	F, 6	F, 12	
Aggression, short tempered	+	+	+	+	+	+	+	+	+	9/9
Attention deficit	−	+	−	+	+	+	+	−	+	5/9
Hypoactive/lethargy	++	++	++	++	++	++	++	++	++	9/9
Bipolar episodes	−	−	−	−	−	−	−	−	−	0/9
Head banging, tantrums, self-mutilation	−	−	−	−	−	−	−	−	−	0/9
Sensitive to food and taste	−	−	−	−	−	−	−	−	−	0/9
Sleep disturbance	−	−	−	−	−	−	−	−	−	0/9
No awareness of clothes	−	−	−	−	−	−	−	−	−	0/9
Thumb sucking	−	−	−	−	−	−	−	−	−	0/9
Unable to attend school	+	+	+	+	+	+	+	+	+	9/9
Self-care (absent)	−	+	+	+	+	+	−	−	+	6/9
Thyroxin withdrawal symptoms present	+	+	+	+	+	+	+	+	+	9/9

+, feature present; ++, severe phenotype; −, feature absent

(Supplemental Table 6; Supplemental Figure 5). There was only one candidate variant, *TPO* c.2315A>G (p.Tyr-772Cys; in exon 12) in a shared homozygosity region of 2.4 Mb at 2p25.3. This variant is rare (only 1 allele in 30616, in South Asian samples) and predicted damaging to the protein by computational algorithms (Supplemental Table 5). It segregated with the disorder in the family (Figure 1; Supplemental Figure 3). It was submitted to ClinVar (VCV000916543.1).

DISCUSSION

In low- and middle-income countries with no newborn screening programs, CH poses a high risk of ID and long-term morbidity. In certain regions of Pakistan, CH is the most frequent inborn error of metabolism with a prevalence of 1/1600 live births [15]. CH is one of the most common treatable causes of ID. Hence, screening programs have been established in most developed countries to detect and treat this disorder, which affects approximately 1 in 2000 to 1 in 4000 newborns [2]. Several studies have evaluated the factors that affect the intellectual outcome in CH [16]. Soe et al. assessed the outcome of LT4 treatment in pediatric patients who were diagnosed as CH in a screening program in Korea [17]. The authors observed that the IQ scores of subjects with early treated CH diagnosed through a neonatal screening test were within the normal range, regardless of etiology, thyroid function, initial dose of levothyroxine, and age at start of treatment. The authors however, remarked that randomized controlled trials with extended datasets are required to establish the optimum dose of LT4 to improve neurologic outcomes.

The manifestation of a broad range of symptoms in certain families with untreated CH poses a big diagnostic challenge particularly in the background of high rates of inherited disorders due to high parental consanguinity. This situation is further aggravated by a remarkably high genetic heterogeneity in ID and neurodevelopmental disorders in Pakistan [18]. Here, ID was observed in two affected members of family 1 and all affected members of family 2. Family 1 had various features not typical of TDHA2 including severe ID with delayed developmental landmarks, speech delay, physical disability, strabismus, enuresis, encopresis, and certain behavioral anomalies. In family 2, the primary presentation was mild to moderate ID in all affected members. In infancy, microcephaly and delayed developmental milestones were reported. Currently, only the youngest affected member (415) has microcephaly. Other features conspicuous in this family were variable verbal apraxia, high myopia, strabismus, frequent faints, infantile epileptic episodes, limb hypotonia with muscle cramps, delayed wound healing, hair loss, sun avoiding behavior, and tooth agenesis. Owing to the wide phenotypic variability, we thus initially launched SNP-based linkage analyses or homozygosity mapping in order to prioritize genomic intervals for subsequent variant evaluation in whole exome data which was complemented with segregation analyses through Sanger sequencing.

To date, more than 120 point mutations (missense, nonsense, or splicing) in the *TPO* gene are listed in the Human Gene Mutation Database and at least 183 in HGMD professional as of August 2023. However, functional effects of most of the identified *TPO* mutations remain unknown [10,19]. Further, there is no obvious genotype-phenotype correlation for *TPO* mutations. In a recent study, Wang et al. carried out screening and functional analysis of four *TPO* mutations in a cohort of Chinese patients with CH and reported that the phenotypes of patients carrying a single heterozygous variant varied remarkably, exhibiting mild, moderate, or severe CH [20]. Even the patients harboring the same mutation had markedly different phenotype and thyroid morphology. Thus, comprehensive studies covering different populations and in-depth functional studies are helpful to have a thorough understanding of the role of *TPO* mutation in the pathogenesis of CH.

ID develops during disease progression if CH goes untreated [4]. To date, ID has been reported to accompany characteristic features of thyroid dyshormonogenesis in only four families. In a consanguineous Pakistani family diagnosed with ID, Iqbal et al. [5] detected a homozygous intragenic deletion of *TPO* exons 11 to 15. Affected members had ID with hypothyroidism but no dysmorphic features. On the other hand, two other Pakistani families reported by Mittal et al. [10] had ID in addition to certain other features including facial dysmorphism. In the family with homozygous c.1786G>T (p.Glu596Ter), affected members had mild to moderate ID, hypothyroidism, delayed development, and dysmorphic facial features. Patients of the second family had homozygous c.1235G>A (p.Arg412His) and hypothyroidism, delayed development of fine motor skills, speech impairment, and dysmorphic facial features. Recently, Fu et al. [19] performed mutation screening of the *TPO* gene in a cohort of 192 Chinese patients with TDH2A. One male patient, 7 years of age, who had ID and developmental retardation harbored triallelic variants, homozygous c.1682C>T (p.Thr561Met), and heterozygous C.1943C>T (p.Arg-648Gln). However, detailed clinical description was not provided. In another Pakistani family with CH and mild ID, a missense variant (c.2315A>G; p.Tyr772Cys) was reported [11].

In comparison with the above-mentioned families with homozygous *TPO* variants and ID, our study families present broad range of symptoms. The features observed in family 2 such as congenital microcephaly, short stature,

high myopia, and dental anomalies were not reported in the published families. Facial dysmorphism is not always evident in untreated TDH2A. Families identified with *TPO*-mutations may be counseled, and screening and preventive therapies may be offered for the other family members and newborns [10]. Taken together, our data suggest that early detection of CH might not be trivial due to the presence of additional symptoms. Furthermore, the early detection and treatment of CH can avoid serious neurodevelopmental consequences, including ID [4].

A prenatal manifestation of CH is fetal goiter which can develop secondary to dyshormonogenesis. These goiters are very small in size and are thus difficult to diagnose at birth and even more challenging during the intrauterine period. However, due to the advances in ultrasound technology, currently diagnosis can be done earlier. Prenatal fetal goiter treatment has been proposed to reduce goiter volume, prevent comorbidities, and provide adequate thyroid status at birth [21]. For the management of CH, intraamniotic T4 injections are recommended in euthyroid pregnant women with large fetal goiters related to hydramnios and/or tracheal occlusion [22].

Patients diagnosed with hypothyroidism require lifelong hormone replacement therapy (Levothyroxine; LT4). Curiously, however, the adherence to treatment among the patients with hypothyroidism is rather poor. A study on Saudi patients revealed that a substantial proportion (67%) of patients with hypothyroidism had low adherence level toward taking LT4, 23% had moderate, and only 10% had high adherence level [23]. A study on Pakistanis reported that patients with hypothyroidism had moderate level of adherence to treatment [24]. In Lebanon, 55% patients had low adherence, 31% medium adherence, and 15% high adherence to therapy [25]. Adherence to hypothyroidism treatment influences the patient's condition including the disease course and outcome.

This study presents the clinical dilemma of untreated CH patients and compromised diagnosis. A broad range of symptoms and atypical phenotypic presentation in our study families is obvious and the delayed identification of etiology leads to irreversible intellectual outcome. ID secondary to CH further adds to the high burden of intellectual disabilities in various consanguineous regions of Pakistan. Secondly, due to the lack of newborn screening program a substantial number of patients are prone to lifelong disability that could have been prevented. Thirdly, its relevance is to reinforce the immediate implementation of newborn screening program for CH in countries with no established programs, particularly in countries with high parental consanguinity such as Pakistan. This study further reveals a long path followed for the diagnosis of CH in two families with a primary presentation of ID and unusual phenotypic presentation. Our detailed clinical assessment complemented with comprehensive molecular genetic analyses including SNP-based homozygosity mapping, linkage analysis, whole exome sequencing, *in silico* analyses of the identified variants led to the final diagnosis of *TPO*-deficiency. These data would be valuable for the family for further carrier screening and genetic counseling.

CONCLUSIONS

TPO-associated ID is clinically highly heterogeneous and the variable symptoms accompanying it could mimic other neurodevelopmental conditions. This situation poses a diagnostic dilemma and is also likely to escalate the disease burden on the society. Our findings highlight the importance of early detection and treatment of CH to prevent severe consequences of untreated TDH2A. We thus propose that newborn screening program for CH should be immediately implemented in all countries. In countries without such a program, infants with a variety of neurodevelopmental symptoms should be evaluated for CH and tested for *TPO* mutation.

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Data availability: The datasets generated and/or analyzed during the current study are available from the corresponding authors on reasonable request.

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Conflict of interest: No financial or non-financial benefits have been received or will be received from any party related directly or indirectly to the subject of this article.

Utilized Data Sources:
ClinVar: https://www.ncbi.nlm.nih.gov/clinvar/
GeneCards: https://www.genecards.org/
GeneDistiller: https://www.genedistiller.org/
MutationTaster: https://www.mutationtaster.org/
Online Mendelian Inheritance in Man (OMIM): https://www.omim.org/
Polyphen2: http://genetics.bwh.harvard.edu/pph2/
REVEL: https://sites.google.com/site/revelgenomics/
SIFT: https://sift.bii.a-star.edu.sg/
UniProt: https://www.uniprot.org/

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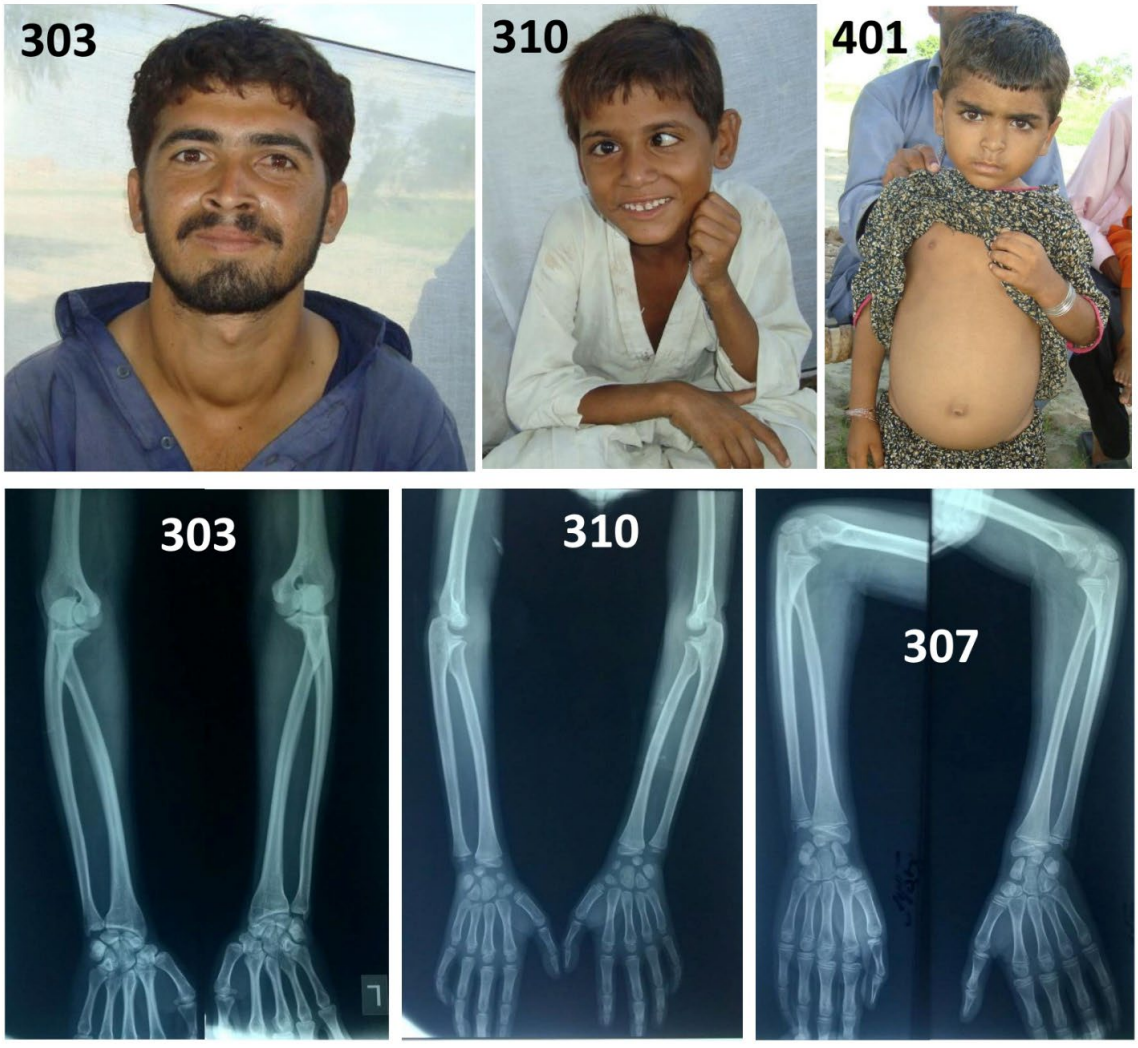
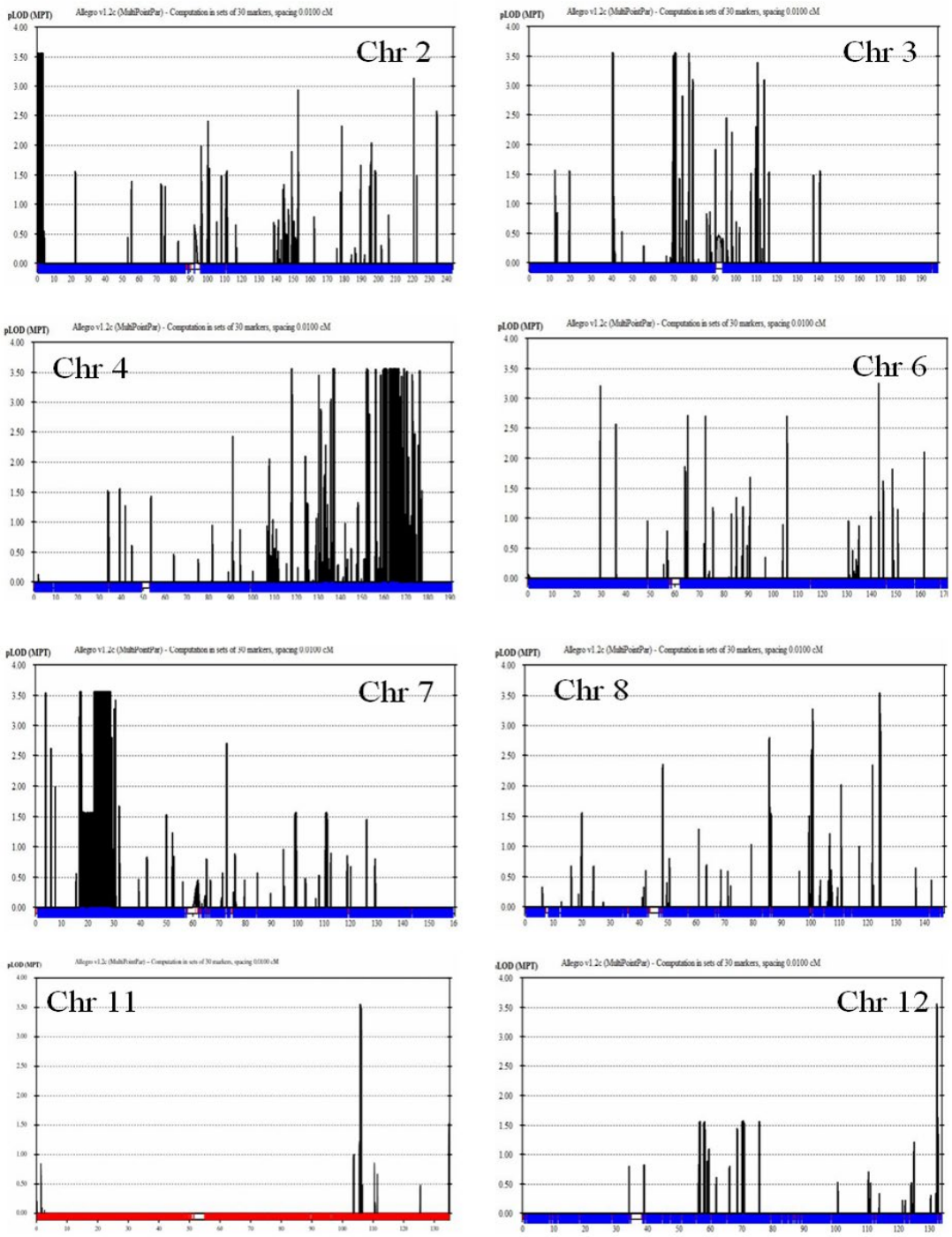
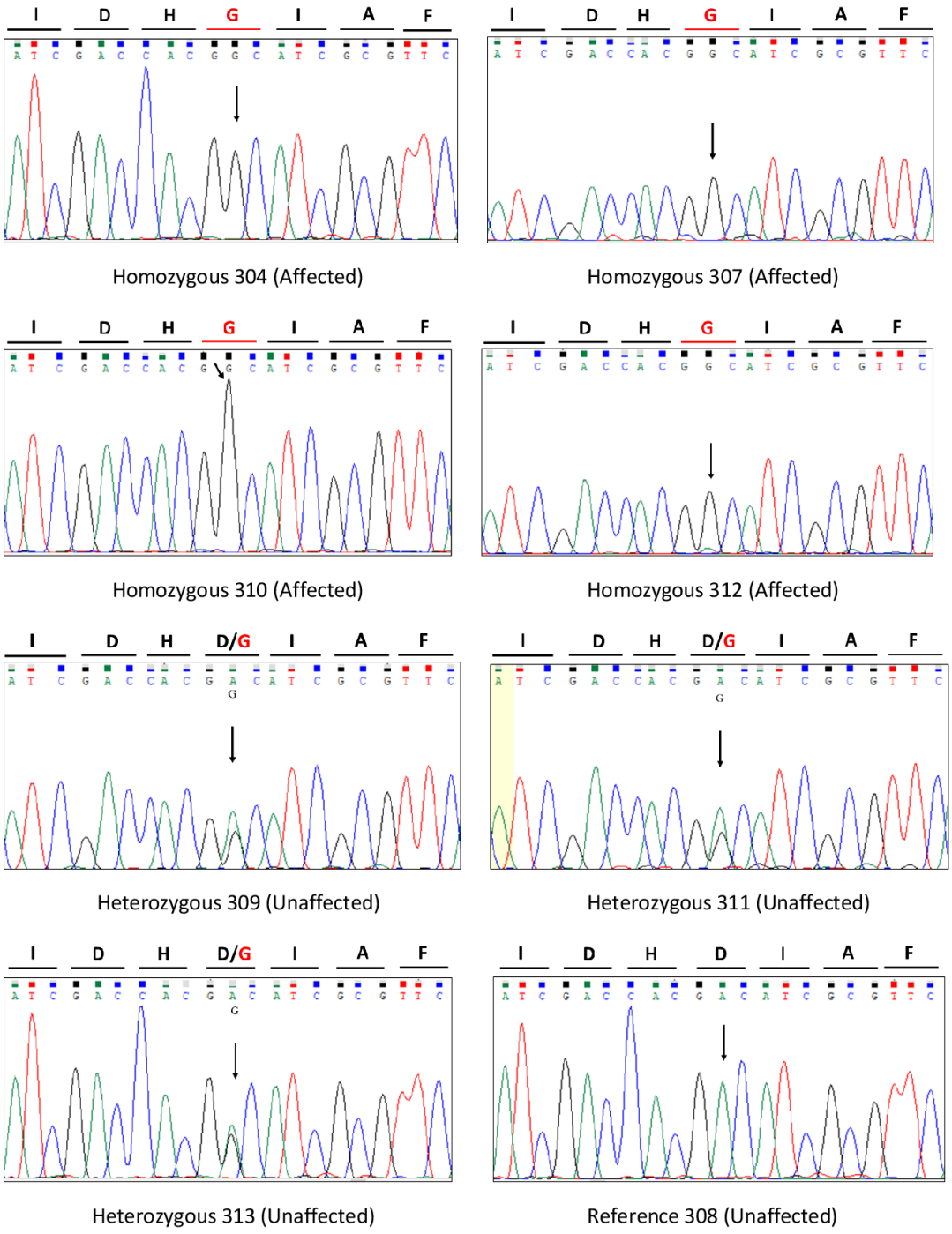
Appendix A



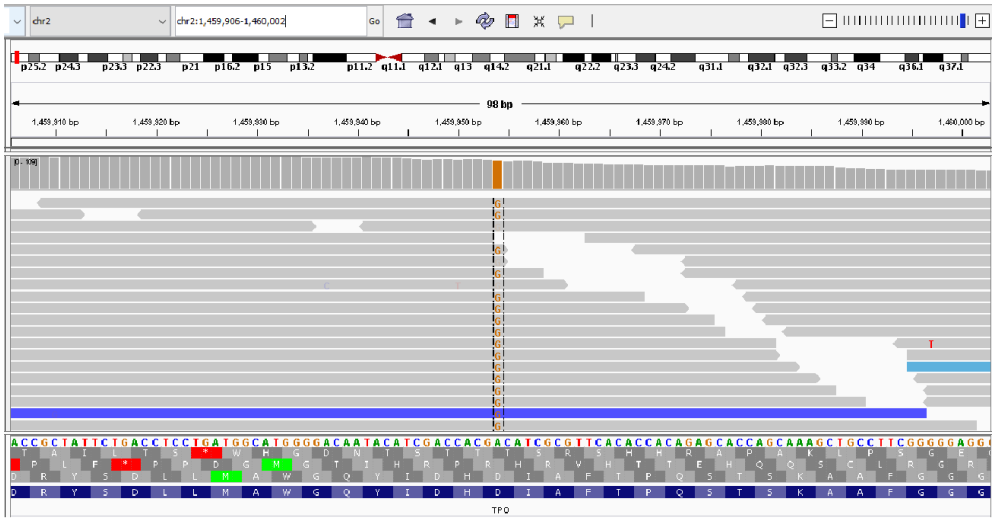
Supplemental Figure 1. Phenotypes of affected members of family 1. Goiter in 303, strabismus and developmental delay in 310, and abdominal swelling and developmental delay in 401. Radiographs of 303, 307 and 310 showing delayed maturation of long bones, hypoplastic carpals and metacarpals, and delayed bone age.



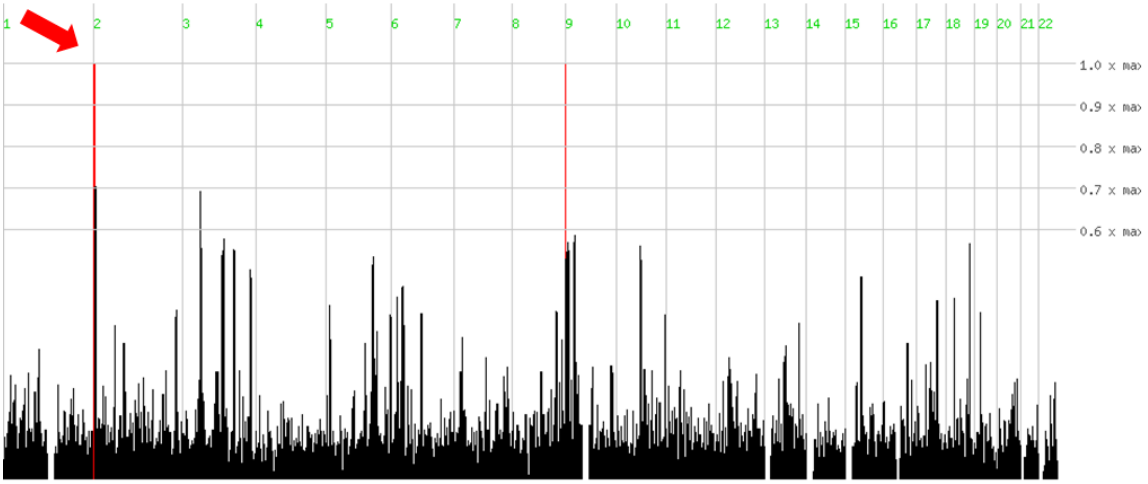
Supplemental Figure 2. Multipoint LOD score graphs of family 1. Only chromosomes with LOD scores >3 are presented.



Supplemental Figure 3. All electropherograms showing the causative variants in *TPO* in families.



Supplemental Figure 4. Display of the mutation *TPO* g.1459954A>G (c.719A>G, p.Asp240Gly) in affected 310 in family 1 on IGV (Integrative Genome Viewer).



Supplemental Figure 5. Homozygosity mapping results in family 2. The largest interval is on chromosome 2.

Supplemental Table 1: Thyroid hormone levels of patients in family 1 and 2

Pedigree ID, sex	Age (yrs) at evaluation	T3 (ng/dl)	T4 (ug/dl)	TSH (ulU/ml)	Free T3 (pg/ml)	Free T4 (ng/dl)
Family 1						
307, F	15	**<25** (80-210)	**1.6** (5.5-12)	**>100.00** (0.50-5)	**< 1.00** (1.71 - 3.71)	**< 0.40** (0.7 - 1.48)
303, M	19	98 (80-210)	**3.7** (5.5-12)	**>100.00** (0.50-5)	2.62 (1.71 - 3.71)	**< 0.54** (0.7 - 1.48)
304, M	19	**220** (80-210)	**5.0** (5.5-12)	**32** (0.50-5)	–	–
	20	145 (80-210)	11.0 (5.5-12)	**0.1** (0.50-5)	–	–
310, M	9	107 (90-230)	8.4 (6-14)	**>100.00** (0.50-5)	3.31 (1.71 - 3.71)	1.15 (0.7 - 1.48)
314, M	0.5	**<20** (85-260)	**0.5** (5.5-16)	**>100.00** (0.6-6.0)	–	–
	6	**28** (90-230)	**1.3** (6-14)	**>100.00** (0.50-5.5)	–	–
Family 2						
405, M	17	125 (80-210)	5.5 (5.5-12)	**9** (0.50-5)	–	–
406, F	15	100 (80-210)	8.4 (5.5-12)	1.8 (0.50-5)	–	–

Reference ranges are in parenthesis. Values in boldface show values deviated from the reference ranges

Supplemental. Table 2: Thyroid ultrasonography results of patients in family 1

Pedigree ID	303	307	310	401
Sex, age (year)	M, 19	F, 15	M, 12	F, 7
Right lobe	Enlarged with multiple masses; largest mass 1.8 x 1.1 cm; cyst of about 1.0 x 0.9 cm; associated vessels are normal	Normal	1.5 cm; mildly enlarged	Normal
Left lobe	Enlarged; 7.1 x 4.2 cm and has a mass of 2.6 x 1.4 cm	Echogenic mass of 11.6 x 13.7 cm detected; associated vessels are normal	1.02 cm	Normal
Impression	Multiple masses in both lobes; cyst in right lobe	Mass in left lobe	Mildly enlarged	Normal

Supplemental Table 3: Radiological imaging of limb bones in affected members of family 1

Pedigree ID	**303**	**307**	**310**
Sex, age (year)	**M, 19**	**F, 15**	**M, 12**
Shortening of long bones	−	+	−
Delayed bone age	−	+	+
Lack of ossification at long bones	−	+	+
Hypoplastic elbow joint	+	+	+
Dysplastic distal head of humerii	+	+	+
Carpals: enlarged capitate; misaligned scaphoid	+	+	+
Hypoplastic carpals and metacarpals	+	+	+
Clinodactyly	−	−	+

Supplemental Table 4: Anthropometric measurements of patients in family 2

Pedigree ID	**401**	**403**	**404**	**405**	**406**	**408**	**411**	**415**	**426**
Sex, age (years)	**F, 25**	**F, 21**	**F, 19**	**M, 17**	**F, 15**	**F, 10**	**M, 15**	**F, 6**	**F, 12**
Standing height (percentile)*	137 (<1)	135 (<1)	132 (<1)	141 (<1)	128 (<1)	102 (<1)	130 (<1)	81 (<1)	139 (<5)
Sitting height	72	79	67	74	71	60	36	36	70
Arms span	131	133	129	144	125	102	132	81	140
Head circumference (percentile)**	52 (<5)	57 (>90)	57 (>90)	58 (>90)	56 (>90)	50 (<5)	53 (<10)	46 (<3)	53 (<50)
Neck circumference	32	36	29	37	28	25	30	25	33
Weight (kg) (percentile)*	32.4 (<1)	51.5 (<25)	29.8 (<1)	48.8 (<5)	30.3 (<1)	15.9 (<1)	NA	NA	35.7 (>25)

All measurements are in cm or if not stated otherwise. NA, not assessed;

*National Center for Health Statistics. Available at: https://www.cdc.gov/growthcharts/.

Rollins, J. D., Collins, J. S. & Holden, K. R. United States head circumference growth reference charts: birth to 21 years. *J. Pediatr.* **156, 907-913.e2 (2010).

Supplemental Table 5. Pathogenicity scores of detected variants as predicted with *in silico* tools.

Variant	c.719A>G (p.Asp240Gly)	c.2315A>G (p.Tyr772Cys)
ClinVar ID	VCV000869101.1	VCV000916543.1
rsIDs	NR	rs1382787497
Prediction tools		
SIFT	Not tolerated	Damaging; 0.001
Polyphen2	Probably damaging; 1.00 (sensitivity: 0.00; specificity: 1.00)	Probably damaging; 0.999 (sensitivity: 0.14; specificity: 0.99)
MutationTaster	disease causing	probable pathogenic
REVEL	Highly damaging; 0.922	Disease causing; 0.221
ClinVar Clinical Significance	NR	Likely pathogenic

NR, not reported

SIFT: https://sift.bii.a-star.edu.sg/

Polyphen2: http://genetics.bwh.harvard.edu/pph2/

MutationTaster : https://www.mutationtaster.org/

REVEL: https://sites.google.com/site/revelgenomics/

ClinVar: https://www.ncbi.nlm.nih.gov/clinvar/

Supplemental Table 6: Intervals (size >2 Mb) of shared homozygosity in family 2. SNP genotypes of a mixture of DNA samples of siblings 401, 404 and 406 plus affected cousin 415 were used.

Chr	Start	End	Flanking rs numbers	Size (bp)
9	44,866,028	67,656,950	rs6606438-rs10908023	22,790,922
9	38,772,575	44,865,026	rs7853023-rs10121167	6,092,451
12	33,778,550	37,625,188	rs7957837-rs11495284	3,846,638
2	18,674	2,388,352	rs10195681-rs13389796	2,369,678