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Original Article

Use of Allele-Specific-Amplification Refractory Mutation System–Polymerase Chain Reaction for the Detection of Thyroid-Stimulating Hormone Receptor Gene Mutation in an Indian Family with Thyroid Dyshormonogenesis

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Abstract

The frequency of thyroid-stimulating hormone receptor (TSHR) gene variant c.1349G>T, p.arginine[R]450leucine[L] in the Asian population has been reported to be high. However, the whole exome sequencing (WES) performed using our samples could not detect this mutation. Moreover, the clinical utility of allele-specific (AS)-amplification refractory mutation system (ARMS)–polymerase chain reaction (PCR) has not been explored in the detection of TSHR gene mutation c.1349G>T, p.R450L in cases with thyroid dyshormonogenesis (TDH). Therefore, we aim to investigate the inheritance and TSHR gene mutation c.1349G>T, p.R450L in an Indian child with TDH and his biological parents. At present, the mutation analysis of TDH-causing genes like dual oxidase (DUOX2) [c.3200C>T, p.Ser(S)1067L] and thyroid peroxidase (TPO) [c.404C>A, p.Pro(P)135His(H)] by WES and TSHR gene variant c.1349G>T/p.R450L by AS-ARMS-PCR was performed. Three years and 1 month-old male child born to nonconsanguineous parents presented with constipation and prolonged indirect hyperbilirubinemia at 1.5 months of age. The evaluation revealed a very high level of serum thyroid stimulating hormone and a low level of free thyroxine (fT4) and fT3. The technetium99 scan showed increased tracer uptake in the thyroid gland. Out of 131 variants identified in TDH-causing genes by WES, DUOX2 gene variant c.3200C>T, p.S1067L and TPO gene variant c.404C>A, p.P135H were noticed to be clinically associated with TDH. Applying AS-ARMS-PCR for the first time, we report the whole Indian family carrying an autosomal heterozygous variant (c.1349G>T/p.R450L) in the hotspot mutation region of the TSHR gene. In conclusion, the known TSHR gene variant c.1349G>T/p.R450L (an autosomal heterozygous), and other variants of the DUOX2 gene (c.3200C>T, p.S1067L) and TPO gene (c.404C>A, p.P135H) might contribute to clinically more severe TDH in children born to carrier parents. We recommend a large-scale study and confirmation of the mutations by Sanger sequencing.

Keywords: Gene mutations, TSH receptor (TSHR) gene, Whole exome sequencing, AS-ARMS-PCR, Thyroid dyshormonogenesis

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Introduction

Defects in any mechanism that interferes with the normal development of the hypothalamic-pituitary-thyroid (HPT) axis [1], thyroid hormones (THs) biosynthesis, action, and its regulation result in congenital TH deficiency, thyroid insufficiency, and thyroid dysfunction **[2,3]**. This autosomal recessive inherited neonatal metabolic endocrine disorder is called congenital hypothyroidism (CH). Phenotypically, CH may be associated with goiter, elevated levels of thyroid-stimulating hormone (TSH) in the case of primary CH, and decreased levels of THs. Without early diagnosis and treatment using levothyroxine (LT4), newborns with CH can face irreversible neurological deficits, severe intellectual disability, mental retardation, long-term metabolic complications, delayed skeletal maturation, and growth retardation [4]. The recorded global incidence of CH has increased from 1:3000–4000 in the initial stage of screening to 1:1000–2000 due to advanced technology, changes in screening algorithms, and increased awareness [5-7]. It occurs with an incidence of 1 in 722 to 1100 births in India [8,9]. The majority of the primary permanent CH cases (80-85%) are attributed to defective development of the thyroid gland or lack of thyroid gland (agenesis; 35–40%) or located in the sublingual position (ectopic; 55–60%) or severely reduced in size though properly placed in the neck (orthotopic hypoplasia; 5%) or the thyroglossal tract and the condition is called thyroid dysgenesis (TD). TD is mainly attributed to mutations in genes encoding for thyroid transcriptional factors (TTF1/NKX2-1, TTF2/FOXE1, PAX8, NKX2-5, TBX1, CDCA8, HOXD3, and HOXB3; [10]) and proteasome (PSMA1, PSMA3, PSMD2, and PSMD3) involved in the development of the thyroid gland and different tissues in the body [11]. Despite the increasing role of genetic factors in CH cases, the genetic defects attributed to TD are far away from being reported. More than 95% of TD cases are believed to be sporadic, and molecular basis has been identified only in about 5% of the cases [12].

However, almost 15–60% of permanent CH cases are believed to be caused by thyroid dyshormonogenesis (TDH) [13], in which biosynthesis of THs and metabolisms of THs are impaired with the eutopic thyroid gland of normal size or goiter [2, 7]. The same thing can be seen on thyroid ultrasonography and varies with the severity of hypothyroidism. The genetic defects in the solute carrier family 5 member 5 (SLC5A5/NIS), solute carrier family 26 member 4 (SLC26A4/PDS), solute carrier family 26 member 7 (SLC26A7), thyroid peroxidase (TPO), NADPH-dual oxidase (DUOX)-1/2, dual oxidase maturation protein (DUOXA)-1/2, iodotyrosine dehalogenase

(IYD/DEHAL1), and thyroglobulin (TG) genes are attributed to TDH affecting the TH biosynthesis at the cell-colloid interface of the polarised follicular lumen of thyroid cells [14]. Most of these mutations are nonsyndromic, familial, and inherited in an autosomal recessive manner, except for the occasional autosomal dominantly inherited DUOX2 gene-based TDH. TDH etiology and natural history are largely extrapolations of CH, and very few studies have specifically addressed TDH in grown children. Moreover, a higher prevalence of mutations in TDH genes (14-91.5%) compared to thyroid dysgenesis (TD) genes (2-21.2%) and thyrotropin resistance genes (9.09-10.9%) have been reported [15-18] to contradict the expected outcomes of ultrasound and scintigraphy [7,19]. Particularly, in patients with normal thyroid gland morphology, it could be very hard to decide radiologically between thyrotropin resistance and TDH or TD.

The mutation in the thyroid-stimulating hormone (TSH) receptor (TSHR) gene causes TSH resistance resulting in increased TSH levels to maintain normal TH secretion which is not always achievable and results in CH [20]. To date, more than 40 distinct loss of function mutations in the TSHR gene from different ethnic backgrounds and geographical origins have been reported [2, 21]. Most of these mutations are reported in exons 1, 2, 6, and 10 of the TSHR gene. Newborn screening approved by most nations determines TSH but not TH concentration, genetically detects only carriers of inactivating biallelic TSHR gene mutations [22] and has a chance to miss autosomal dominant-inherited monoallelic variants with milder symptoms. The genetic heterogeneity associated with different clinical phenotypes and different forms of CH is not well understood, especially in the Indian context, where the birth rate, consanguinity, and prevalence of TDH are higher (>20%). The pathogenic factors contributing to TDH cases in India may be different from those in other populations. All the exons and introns of three candidate genes (TSHR, TPO, and TG) were not analyzed by only a study from India and hence there is a chance of missing rare variants associated with CH [23]. Moreover, the prevalence and incidence of TSHR gene mutation at c.1349G loci in the Asian population have been reported to be high compared to other populations [20, 24]. The mutationc.1349G>T,p.arginine[R]450leucine[L] in the Indian population was not investigated, though it has a significant effect on TSHR functioning and triggering TDH. Therefore, we aim to investigate the inheritance and TSHR gene mutation c.1349G>T, p.R450L in an Indian child with TDH and his biological parents.

Materials and methods

Study design

A prospective case study was set in the Genetic Research Centre (GRC), ICMR-NIRRCH, Mumbai. The study protocol and all procedures were approved by the Institutional Ethics Committee (IEC) of ICMR-NIRRCH, Mumbai (D/ICEC/Sci-111/116/2022 dated 22-07-2022) and also by the Collaborating Centre (Bai Jerbai Wadia Hospital for Children (BJWHC; IEC-BJWHC/07/2022 dated 29-01-2022). The routine neonatal check-ups of the neonates and children were done at BJWHC, Mumbai. Clinically diagnosed child with TDH and his biological parents identified by the clinician

were enrolled after obtaining consent and assent as per the inclusion and exclusion criteria. Selection of the child with TDH and his biological parents for this study was done based on the following inclusion and exclusion criteria:

- The inclusion criterion for the child includes a child diagnosed with TDH by hormonal profile/thyroid scanning
- The exclusion criteria for the child include 1) a child associated with congenital adrenal hyperplasia, hyperaldosteronism, pituitary disorders, and hyperthyroidism, 2) a child with thyroid autoimmune disease, 3) maternal use of antithyroid drugs during pregnancy, 4) child received of blood transfusion within the prior 2 months of enrolment and/ or at the time of blood collection, 5) the child is on a formulated diet and has evidence of severe infections, 6) a child with significant comorbidities like adrenal insufficiency, growth hormone deficiency, gestational Diabetes mellitus (GDM), cancer, and other autoimmune diseases (rheumatoid arthritis, systemic lupus erythematosus, and multiple sclerosis), and 7) the child of one or both parent(s) is/ are not available or not willing to enroll herself/ himself or themselves.
- The inclusion criterion for parents of a child with TDH includes 1) must be biological parents of a child with TDH (mother and father), 2) the parents of a TDH child with a history of CH and symptoms of TDH
- The exclusion criteria for parents include 1) mother/ father associated with endocrine abnormalities other than CH, 2) mother/ father with thyroid autoimmune diseases, 3) Parents with significant comorbidities like diabetes, cancer, and other autoimmune diseases (rheumatoid arthritis, systemic lupus erythematosus, and multiple sclerosis), 4) parents with evidence of severe infections in the past, 5) one or both parent(s) of a child is/ are not available or not willing to enroll herself/ himself or themselves.

The socio-demographic details like gender, age at the time of diagnosis, birth weight, gestational age, mother's age at delivery, co-occurring congenital anomalies, maternal history of thyroid disorder, parental consanguinity, and birth order were recorded along with the technetium99 scanning, and levels of serum TSH, free thyroxine (fT4) and fT3. Details of comorbidities like adrenal insufficiency, growth hormone deficiency, GDM, cancer, and other autoimmune diseases (rheumatoid arthritis, systemic lupus erythematosus, and multiple sclerosis) have been ruled out after performing suitable procedures. Clinical diagnosis of thyroid autoimmune disease was performed by the clinician based on the case history, physical examination, laboratory diagnosis, and previous reports of thyroid antibody tests/ Iodine urine tests, or thyroid stimulating immunoglobulin tests. Definitive diagnosis using WES and further AS-ARMS-PCR was performed in the following manner to confirm the actual cause of the TDH.

Blood collection and DNA extraction

Two (2) mLs of whole blood in the K2, EDTA (3.6 mg, BD Vacutainers, BD Catalog no: 367899) from the child with TDH and his biological parents were collected. Samples collected only once from the family along with routine other tests were brought to GRC, NIRRCH, Mumbai with brief clinical details.

Table 1: The list of genes known to cause congenital hypothyroidism (CH)

AADAT	DUOX1	GNAS	KMT2D	POU1F1	RYR2	SLC30A10	THRB	TTR
ALB	DUOXA1	HHEX	NCOR2	PROP1	SALL1	SLC5A5	TPO	TUBB1
ALMS1	DUOX2	HOXA3, HOXD3, HOXB3	NKX2-1/ TTF1	PSMA1	SECISBP2	SLCO1C1	TRH	UBR1
ATXN2	DUOXA2	IGSF1	NKX2-5	PSMA3	SERPINA7	STAMBP	TRHR	VAV3
CDCA8	EXOSC2	IRS4	NKX2-6	PSMD2	SLC16A2	TBL1X	TRIP11	
DIO1	FGF8	IYD	NTN1	PSMD3	SLC17A4	TBX1	TRIP12	
DIO2	FOXE1/ TTF2	JAG1	P4HB	PTH1R	SLC26A4	TG	TSHB	
DIO3	GLIS3	KDM6A	PAX8	PTRH2	SLC26A7	THRA	TSHR	

The genomic DNA isolation from 200µl of whole blood was carried out using a commercially available QIAamp DNA Blood Mini Kit (Catalog no. 51104, QIAGEN, www.qiagen.com). The quantity of the genomic DNA (ratio at 260/280nm) was assessed using the Nanodrop technique (BioTek Synergy H1 Multi-Mode Reader), and Qubit (Thermo Scientific, USA). The concentration of the genomic DNA thus obtained was approximately 46.66 ng/µl for the father, 43.56 ng/µl for the mother, and 19.91 ng/µl for the child with TDH. The genomic DNA (5 µl) with 1 µl of ExcelDye™ 6X DNA Loading Dye (DL4000, Tri-color by SMOBIO Technology, Inc) was loaded on 0.8% agarose gel (Cat no. FB0010 (500g); Bio Basic) incorporated with ethidium bromide. The gel electrophoresis was carried out at a voltage of 80V for 1 hour using 1X TAE buffer on an agarose gel electrophoresis unit (Bangalore Genie, Bangalore, India). The bands in the gel were photographed under a Bio-Rad Launches ChemiDoc™ MP Imaging System (Catalog no: 12003154). The purity/ quality and size of the genomic DNA were determined by comparing the relative intensities of the bands and the distance they migrate with the DL5000-DNA molecular weight marker (Cat no. 3428A, Takara Bio companies). The extracted genomic DNA from the child with TDH was sent to Genotypic Technology [P] Ltd, Bangalore, India for whole-exome sequencing (WES).

Whole-exome sequencing (WES)

Briefly, 100 - 200 ng of Qubit quantified genomic DNA of a child with TDH was sheared by adaptive focused acoustics using a Covaris S220 system (Covaris, Woburn, Massachusetts, USA). The fragment size distribution was verified on Agilent 2100 Bioanalyser. The fragments were end-repaired, adenylated, and ligated to Illumina adaptors as per the SureSelectXT library preparation kit protocol. WES of the genomic DNA of a child with TDH generated ~27.3 million reads with sequencing coverage of 136X was carried out.

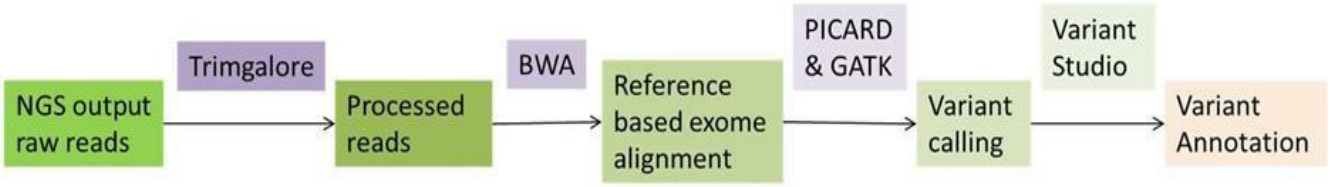

Figure 1: Bioinformatics workflow of whole exome sequencing

The adapter free good quality processed reads were aligned against the reference human genome (hg19/GRCh37) for variant prediction. The raw variants were filtered with standard filtering criteria (minimum read depth of 20 and mapping quality of 30) before functional annotation. Annotation details were retrieved for the variants using various clinical databases along with their population frequencies. The complete bioinformatics analysis workflow is given in **Figure 1**. Based on anticipated functional scores, allele frequency, and zygosity, mutational analysis of thyroid genes linked to CH (**Table 1**) was reviewed and potential variants connected to the trait were identified. All discovered variants were then assessed using Sorting Intolerant from Tolerant (SIFT) [25], Polymorphism Phenotyping (PolyPhen) [26], and Combined Annotation Dependent Depletion Score (CADD) [27] prediction scores to determine how harmful the discovered variants were noted.

Table 2: Details of the allele-specific primers and amplicon size in base pairs (bps) on AS-ARMS–PCR

Genotype/ product	Forward primer	Reverse primer	Annealing	Amplicon size (in bp)
T-allele specific	TSHR OFP:5'-TACCCCCAAGTCCGATGAGT-3' (Size=20 bp, Tm=59.35ºC, GC content=55%, no G/C at 3'end, no hairpin loop formation and dimerization found)	TSHR IRP: 5'-CAGGTTGCACATGAGAAAGT-3' (Size=20bp, Tm=55.25ºC, GC content=45%, no G/C at 3'end, no hairpin loop formation and dimerization found)	50 ºC	175 bp
G-allele specific	TSHR IFP: 5'-ACAAACTGAACGTCCCCCG-3' (Size=19 bp, Tm=58.82ºC, GC content=57.89%, G/C at 3'end, no hairpin loop formation and dimerization found)	TSHR ORP: 5'-CCTGGGTTGTACTGCGGATT-3' (Size=20bp, Tm=59.35ºC, GC content=55%, no G/C at 3'end, no hairpin loop formation and dimerization found)	62 ºC (in-house prepared master mis) and 65 ºC (2X supperphlex master mix)	517 bp
Internal control	TSHR OFP	TSHR ORP		654 bp

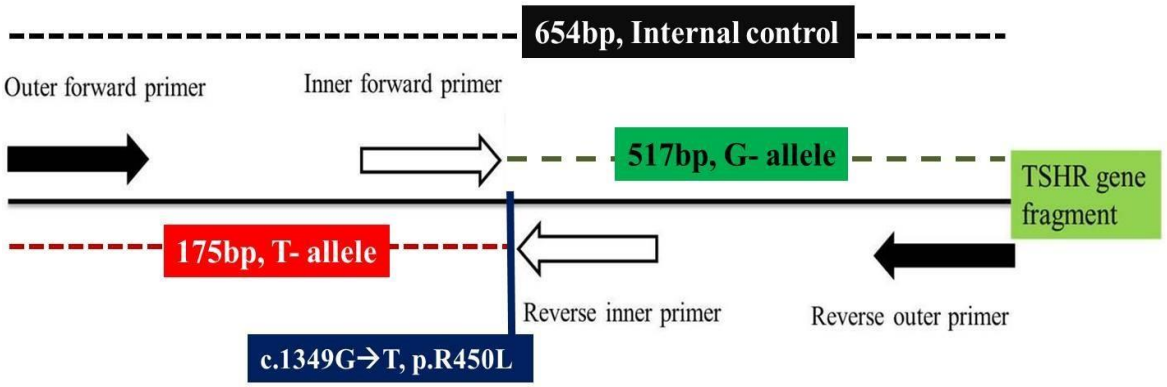

Figure 2: A schematic representation of primers covering the TSHR gene variant c.1349G>T, p.R450L for ARMS–AS-PCR and amplification pattern

Allele-specific (AS)-Amplification refractory mutation system (ARMS)–polymerase chain reaction (PCR)

Primer designing, validation, and PCR optimization:

The NCBI CCDS database was used to obtain the sequence of the TSHR gene. A schematic presentation of primers covering the c.1349G>T, p.R450L site of the TSHR gene was given in **Figure 2.** Primers were designed manually as per the ideal parameters of primer designing guidelines. The specificity and suitability of primers for PCR were checked using the Insilico PCR online tool (https://genome.ucsc.edu/cgi-bin/hgPcr). Sequence manipulation suite for PCR Primer Stat tool (https://www.bioinformatics.org/sms2/pcr_primer_stats.html) was applied to check general properties of the primers like quality, GC content, melting temperature, etc. The primers showing sequence similarities with imprecise target regions in the genome were avoided. Details of the allele-specific primers, sequence, and amplicon size in base pairs (bps) on AS-ARMS–PCR were given in **Table 2**. NCBI- Nucleotide Blast https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome was applied to confirm the specificity of the designed primers. The target-specific amplification of genes using the right primers with appropriate primer size (18–28 bases), high GC content (50-60%), higher melting temperature (Tm =[2 (A+T)+4 (G+C)]; 55–80 °C), 3' end of primer with G/C or CG/GC, primers without intra /inter complementarity (failure to form 2^o structures), primers without the runs of three or more Cs/Gs at the 3'ends, and amplicon with a varied length (between 150 base pairs [bp] and 1000 bp) to discriminate on agarose gel electrophoresis were applied. The optimal annealing temperature (usually starts at 3–5°C below the lowest Tm of the primer; 52–70 °C), an equal number of all primer molecules (number of molecules = [concentration of the primer * Avogadro number] / number of base pairs of primers * 650 (average weight of one nucleotide in Daltons) * 10^9; http://cels.uri.edu/gsc/cndna.html) and an equal concentration of the template genomic DNA (19.91- 46.66 ng/µl) were used to amplify the targeted region of the genomic DNA. The minimum time for a reaction to happen at each step in the case of PCR with more cycles and two loops (each one with 25 cycles) touchdown PCR with two different annealing temperatures was applied for successful amplification of the multiple targets. Maximum reaction volume for better resolving the target amplification was applied. The higher denaturation (98^oC) and higher annealing temperature to avoid non-specific amplification and erratic efficacy on diverse templates were applied for a successful AS-ARMS–PCR. The competition, cross-hybridization, short amplification efficiency, mispriming, and/or mispairing with other non-specific products due to the existence of complexity and several primers in a reaction mixture were avoided [28].

Primer synthesis, reconstitution, and quality check

Once the primers were verified for their suitability using the Insilico online tool, the sequences were sent to Eurofins Genomics India Pvt Ltd for synthesis. Primers were obtained in the lyophilized form and were

reconstituted according to the manufacturer's instruction using freshly prepared and autoclaved 1X TE buffer (1 mM Tris, 10 mM EDTA, pH 8.0) and stored at - 20^{o}C. The main stock concentration of each primer was 100 pmol/µl. Further, each primer was diluted to 10 pmol/µl for working stock. The primers from all stocks were checked on 2% agarose gel electrophoresis for their quality and intactness (Supplementary **Figure S1**). Electrophoresis was carried out at a constant voltage (80V) for 45 minutes at room temperature in 1X TAE buffer using an agarose gel electrophoresis unit with a universal power pack (Bangalore Genie, Bangalore, India). The bands in the gel were photographed under a Bio-Rad Launches ChemiDoc™ MP Imaging System (Catalog no: 12003154) and documented.

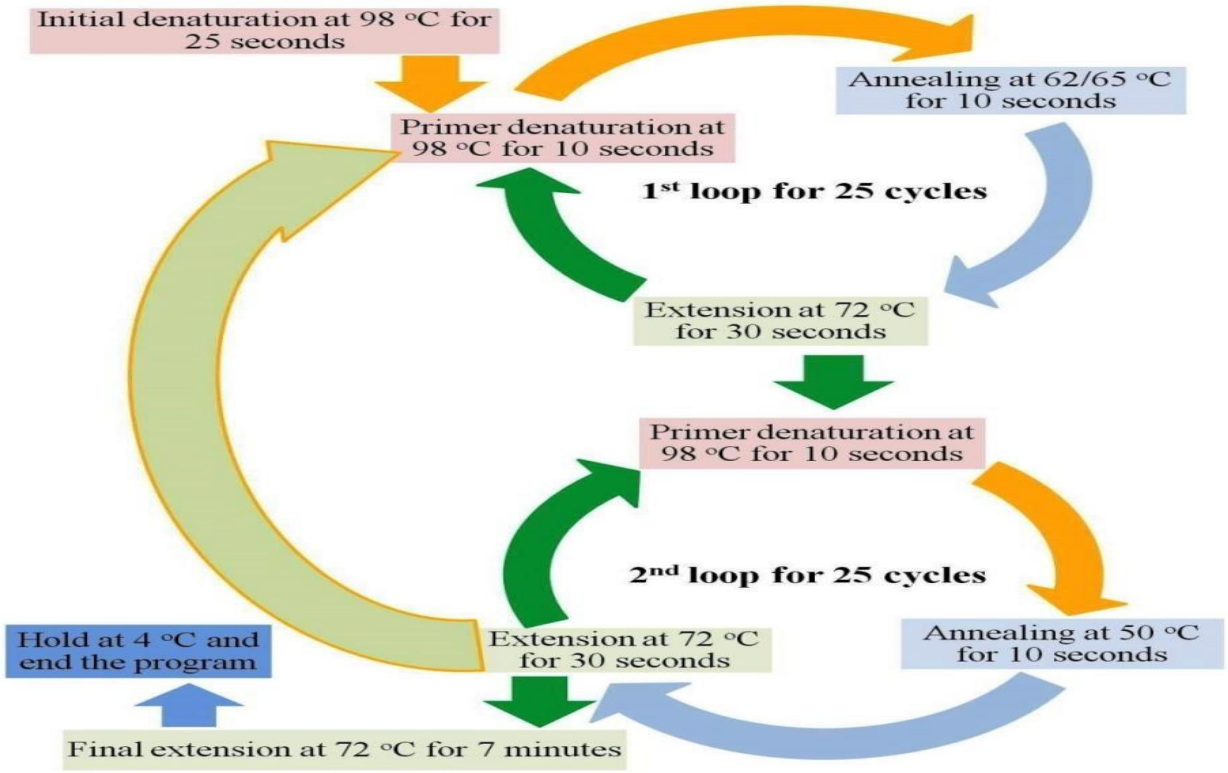

Figure 3: A schematic representation of two looped touchdown AS-ARMS-PCR programs. 1st loop at 62^{o}C using an in-house prepared master mix and 65^{o}C using 2X supperphlex master mix for 25 cycles. 2nd loop at 50^{o}C for 25 cycles.

PCR optimization and determination of family genotype for TSHR gene

All reaction components were kept on ice before starting the experiment. PCR optimization using different template genomic DNA with different concentrations, a different set of primers with different concentrations, and a different number of primer molecules using an in-house prepared master mix [10X Taq Buffer (Catalog no: R001A); dNTP mixture (2.5 mM), Taq™DNA Polymerase (250U); Takara] and 2X SuperPlex Premix (Catalog no: 638543 Takara Bio companies, USA) at different annealing temperatures (ranging from 50-67^{o}C) with different reaction time was carried out. The components to the PCR tubes were added by following the order given in Supplementary **Tables S1** and **S2**. Confirmation of the PCR amplification at 50 ^{o}C annealing temperatures for T-allele (175bp), and 65 ^{o}C annealing temperatures for G-allele (517 bp) and internal control (654bp), respectively using all sets of primers (1µl of 10 pmol/µl of each primer), 2x Superplex master mix (12.5µl) and different template genomic DNA in the single tube-single reaction (volume with 25µl) was carried out. The amplified products of PCR (5µl) along with 6X loading were loaded onto the 1.5% agarose gel electrophoresis and bands in the gels were verified and analyzed on a Bio-Rad Launches ChemiDoc™ MP Imaging System (Catalog no: 12003154).

Confirmation of the genotype using the optimized reaction and PCR program: Three PCR reaction tubes (0.2 ml sterile microfuge tubes) were labeled; one for the child, one for the mother, and another for the father. The components to the PCR tubes were added by following the order given in Supplementary **Table S2**. The reaction mixture includes the template genomic DNA of the child with TDH (19.91ng/µl; in the 1st tube), the template DNA of the mother of the TDH child (43.56 ng/µl; in the 2nd tube), the template DNA of the father of the TDH child (46.66 ng/µl; in the 3rd tube), two sets of primers (10 pmol/µl each) in each tube and 2X SuperPlex Premix in each tube. The reaction mixtures were mixed gently by vortexing and briefly centrifuged to sediment all the components to the bottom of the tube. These PCR tubes were transferred from the ice onto a PCR thermal cycler (SureCycle 8800, Agilent Technologies, United States) with the block preheated to 98 °C to begin thermocycling. The template genomic DNA was initially denatured at 98 ^{o}C for 25 seconds, then primers denatured at 98 ^{o}C for 10 seconds, annealing at 65 ^{o}C for 10 seconds (1st loop for 25 cycles), and 50 ^{o}C for 10 seconds (2nd loop for 25 cycles) and extended at 72 ^{o}C for 30 seconds (details of the PCR program is given in **Figure 3**). Finally, 7-minute incubation at 72 ^{o}C was carried out to complete the partially synthesized second strands. Upon completion of PCR, samples were stored at 4 ^{o}C. The amplified products of PCR (5µl) were mixed with 1µl of 6X DNA loading dye and subjected to electrophoresis on a 1.5% agarose gel incorporated with ethidium bromide in 1X TAE buffer along with the 100bp DNA molecular weight marker. The electrophoresis was carried out at a constant voltage (80V) for 1 hour at room temperature in 1X TAE buffer

using an agarose gel electrophoresis unit with a universal power pack (Bangalore Genie, Bangalore, India). The bands in the gel were visualized and quantified under ultraviolet illumination (Bio-Rad Launches ChemiDoc™ MP Imaging System; Catalog no: 12003154).

Ethical considerations

Ethics approvals were obtained from ICMR-National Institute for Research in Reproductive and Child Health (D/ICEC/Sci-111/116/2022 dated 22-07-2022) and also from Bai Jerbai Wadia Hospital for Children, Mumbai (IEC-BJWHC/07/2022 dated 29-01-2022).

Written informed consent was obtained from the participants for the genetic analysis and publication of their reports. A copy of the written consent will be made available at the request.

Results

Three years and 1 month-old male child born to non-consanguineous biological parents with a birth weight of 3200 g was recruited. The serum TSH concentration on the first day of life was normal i.e., 7.44 µIU/mL (normal range: 2.69-26.5 µIU/ mL) and the serum free thyroxine (fT4) was 1.13 ng/dL (normal range: 1.2-2.6 ng/dL). Family history was normal for thyroid problems except for the mother who had gestational hypothyroidism. The child first presented with constipation and prolonged indirect hyperbilirubinemia at 1.5 months of age. The evaluation revealed a very high level of TSH (>60 µIU/mL; normal range: 0.58-5.57 µIU/mL) and a low level of fT3 (1.45 pg/mL; normal range: 2-4.4 pg/mL) and fT4 (0.57ng/dL; normal range: 1.04-3.4 ng/dL) after treatment. The ultrasonography for the thyroid gland was normal (right lobe-1.2*1.1 cm, left lobe-1.2*1.3*1.1 cm, isthmus 2 mm). The technetium99 scan showed increased tracer uptake in the thyroid gland (total thyroid uptake was 9.8%; normal range: 1-5%). A preliminary diagnosis of CH likely due to TDH was made and the child was started with 37.5 µg of levothyroxine (T4). The confirmatory diagnosis was made by understanding the genetic basis of the condition and its pattern of inheritance. To understand the genetic basis of the condition, the design and selection of the specific set of primers were vital for a successful AS-ARMS–PCR. The wild-type nucleotide sequence of the TSHR gene was identified using the Open NCBI CCDS database. A total of 2295 nucleotides obtained were used for designing primer covering the c.1349G>T, p.R450L site on the TSHR gene. Four primers (two outer primers and two allele-specific inner primers) meant to amplify three amplicons of different sizes of the TSHR gene on AS-ARMS-PCR were designed. All three products specifying the size, localization, and alignment with the target regions of the THSR gene located on chromosome 14q31 were identified, thus confirming the specificity of the primers. Optimization of the AS-ARMS-PCR was divided into several steps. First, a single annealing temperature to amplify multiple targets on a single tube-single reaction PCR was optimized. Only T-allele-specific bands at 50^{o}C annealing temperature on the gradient ARMS-PCR were observed. Hence, an independent PCR program to amplify all loci individually using each set of target-specific primers at 50^{o}C, 62^{o}C, and 65^{o}C of annealing temperature was performed. Outer forward primer and T-allele specific inner reverse primer were able to

amplify T-allele specific (mutated) product of 175 bp at 50ºC annealing temperature (Supplementary **Figure S2)**. Similarly, G-allele specific inner forward primer and outer reverse primer amplify the G-allele specific (wild type) product of 517 bp, and outer forward primer and outer reverse primer amplify the internal control (full TSHR gene) product of 654 bp at 62 ºC annealing temperature using an in-house prepared master mix were noted (Supplementary **Figures S3**).

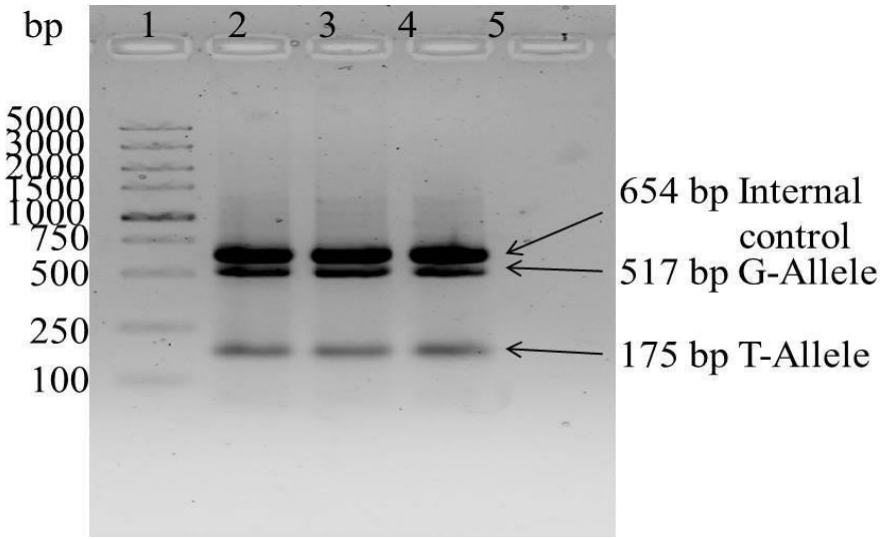

Figure 4: The two looped touchdown-AS-ARMS-PCR amplified products at 65ºC annealing temperature (1st loop) and 50ºC (2nd loop) on the 1.5% agarose gel electrophoresis. Well 1- DNA Ladder. Well 2- bands specifying T-allele (175 bp band from bottom), G-allele (517 bp, in the middle), and internal control (654 bp, on the top) using template DNA of child with TDH, Well 3-bands specifying all three amplicons using template DNA of mother of the affected child and Well 4- bands representing all three amplicons using template DNA of the father of the affected child were amplified using all sets of primers in a single tube –single reaction. Well 5- Negative control.

Table 3: Details of demographic, clinical, and two looped touchdown-AS-ARMS-PCR findings of TSHR gene variant c.1349G>T in the child with TDH and his biological parents

Characteristics	Gender	Age	Clinical presentation	Internal control (654 bp)	PCR for c.1349G>T, p.R450L		Genotype & location
					G-Allele (517 bp)	T-Allele (175 bp)	
Child with TDH	Male	3 years 1 month	Constipation and history of prolonged neonatal jaundice	Yes	Yes	Yes	Autosomal heterozygous c.1349G>T, p.Arg450Leu
Mother	Female	28 years	Gestational hypothyroidism	Yes	Yes	Yes	Autosomal heterozygous c.1349G>T, p.Arg450Leu
Father	Male	33 years	Normal	Yes	Yes	Yes	Autosomal heterozygous, c.1349G>T, p.Arg450Leu

All products specifying T-allele (mutated), G-allele (wild type), and internal control using an individual set of primers and all sets of primers in a single run were noted using template

genomic DNA (of the child with TDH and his parents) and in-house prepared master mix at 62 ^{o}C annealing temperature and 2X supperphlex at 65 ^{o}C annealing temperature. However, a light band specifying T-allele (mutated) using all sets of primers and a single set of primers at 62^{o}C using an in-house prepared master mix and 65^{o}C using 2X supperphlex was observed. On further repetition, the better amplification of the T-allele specific product at a lower annealing temperature (50^{o}C), and the G-allele specific product and internal control at a higher annealing temperature (62 & 65 ^{o}C) were noted (**Figure 4**). Hence, the concentrations of all sets of primers and template genomic DNA were equalized and a two-looped touchdown AS-ARMS-PCR protocol (1st loop with 65 ^{o}C for 25 cycles and 2nd loop with 50 ^{o}C for 25 cycles) was implemented. Amplicons (DNA bands) representing T-allele, G-allele, and internal control without nonspecific amplification were detected in the whole family with improved quality using the AS-ARMS-PCR protocol. The child with TDH is an autosomal heterozygous for germline missense variants of the TSHR gene (NM_000369.5:c.1349G>T, R[CGC]>L[CTC], NP_000360.2:p.Arg450Leu, rs189261858) with a known phenotype of TDH and may be associated with TSH resistance. Thymine replaces the ancestral allele (guanine), which results in an amino acid exchange from arginine to leucine (p.Arg450Leu). Mother and father were found to be carriers of this mutation (an autosomal heterozygous). This study reports that the TSHR gene variant c.1349G>T was identified in the whole family by AS-ARMS–PCR. Details of demographic, clinical, and two looped touchdown-AS-ARMS-PCR findings of TSHR gene variant c.1349G>T in the child with TDH and his biological parents were given in **Table 3.**

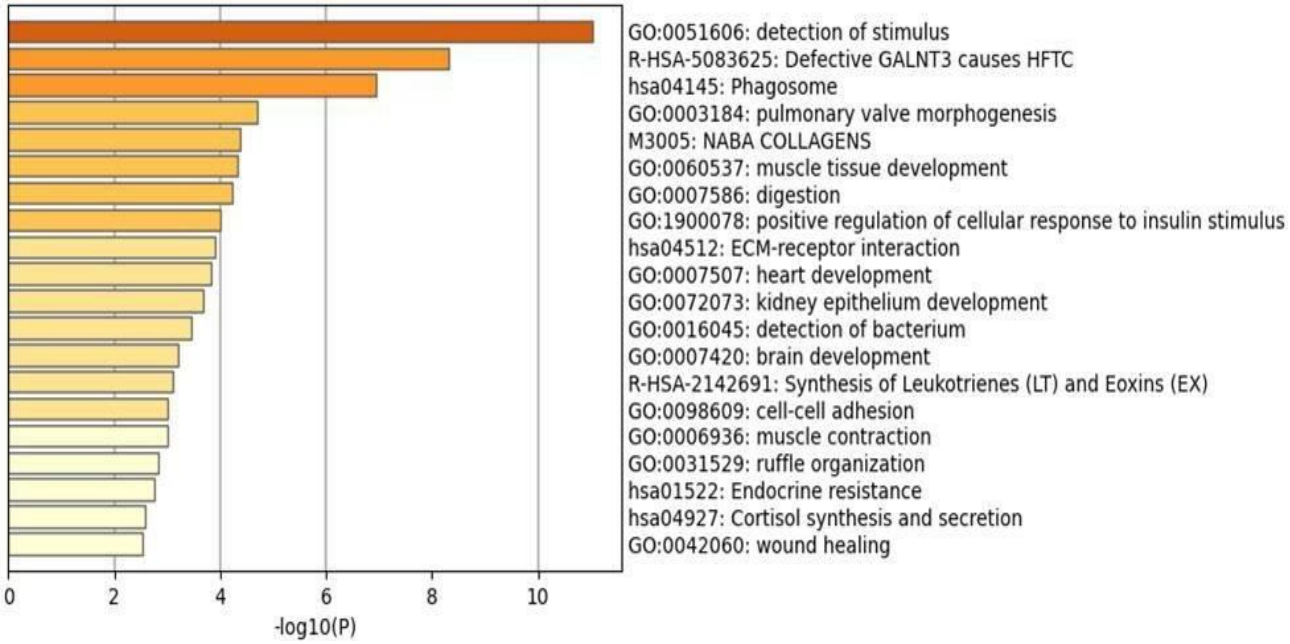

Figure 5: Metascape Bar graph of enriched terms across input gene lists, colored by p-values

Table 4: Findings of the whole exome sequencing (WES) and two looped touchdown-AS-ARMS-PCR in the child with TDH.

Gene	Position	Exon	Change in DNA sequence (CDS)	Aminoacid change in the protein	Sift/ PolyPhen	ACMG Pathogenicity classification	Zygosity	Inheritance	ClinVar / gnomAD/1000 genome GMAF	Depth and CADD score >20	dbSNP ID	ClinVar RCV Id:	Others
DUOX2	SNV:15-45392075-G-A(GRCh37)	25	c.3200C>T	p.Ser(S)1067Leu(L)	Deleterious (0.04)/benign (0.024)	Benign	Hom	AR	0.27975	yes	rs269868	RCV000607232.7	MedGen: C1846632; Orphanet: 95716; OMIM: 607200
TPO	SNV:2-1440078-C-A(GRCh37)	4	c.404C>A	p.Pro(P)135His(H)	Deleterious (0.01)/ damaging (0.927)	Benign/likely benign/VUS	Het	AR	0.00879	yes	rs61758083	RCV000334710.5	MedGen: C1291299; Orphanet: 95716; OMIM: 274500
TSHR	SNV:14-81609751-G-A(GRCh37)	10	c.1349G>T	p.Arg(R)450Leu (L)	Nil	Likely pathogenic, benign	Het	AR	0.0002	AS-ARMS-PCR	rs189261858	RCV000490528.5	MedGen: C3493776; Orphanet: 90673; OMIM: 275200;

Note: AR, Autosomal recessive; hom, homozygous; het, heterozygous; AS-ARMS-PCR, Allele-Specific-Amplification Refractory Mutation System–Polymerase Chain Reaction, VUS, variant of unknown significance.

The WES of the genomic DNA of a child with TDH generated a total of 60121 variants of TDH-causing genes and genes unknown to cause TDH. Out of which, the variants with global rare allele frequency (RAF) < 0.05 were 12731. The variant prioritization criteria and the number of variants prioritized are given in Supplementary **Table S3.** Out of the unknown category of gene variants to cause TDH, only 409 variants with potential transcripts were identified. A comprehensive gene list annotation and analysis resource using 409 variants of unknown categories was captured using Metascape (http://metascape.org). Biological processes (GO) enrichment analysis was applied to each MCODE network to extract "biological meanings" from the network component, where the top three best p-value terms were retained (Supplementary **Table S4**). This analysis points towards the detection of stimulus (GO:0051606) and hyperphosphatemic tumoral calcinosis (HFTC) due to defective N-acetylgalactosaminyltransferase-3 **(**GALNT3; R-HAS-5083625**; Figure 5)**. In the TDH-causing genes category, a missense variant in the DUOX2 gene by a transversion of T to C in exon 24, which replaces serine with leucine at codon 1067: c.3200C>T, p.S1067L and TPO gene mutation c.404C>A in exon 4, which replaces proline with histidine at codon 135: c.404C>A, p.P135H demonstrating harmful and benign/damaging effect, respectively on SIFT and PolyPhen prediction were detected out of 131 variants discovered by WES. Out of eight variants in the TSHR gene, one autosomal homozygous missense variant at 2181CDS position (10^{th} exon) with altered amino acid-glutamic acid (E) / aspartic acid (D) due to the replacement of ancestral allele guanine (G) with cytosine (C) was noted on examination of WES

data of the child with TDH. According to the significance of the gnomAD and clinvar databases, variants were categorized as either pathogenic or likely pathogenic, variants of unclear significance (VUS), likely benign, or benign (**Table 4**). Because Sanger sequencing is insensitive to small populations and may miss mosaicism and a variety of deletions or duplications in the same sample, it was not recommended. Furthermore, at 700 to 900 bases, sequence quality starts to decline. If this strategy is not used early on in the diagnostic work-up, delays in diagnosis and improper treatment may result [29]. However, the confirmation of variants by Sanger sequencing helps in identifying patterns of inheritance.

Discussion

Thyroid dyshormonogenesis (TDH) is known to manifest with goiter. Thyroid ultrasonography shows eutopic thyroid and depending on the severity of hypothyroidism, the thyroid size may be normal or large/goiter. Clinically, there is fluctuation in the TSH, T4, and T3 levels. In our study, scintigraphy revealed a high thyroid uptake that may have been caused by an overstimulated gland with elevated TSH levels or by the stimulation of NIS expression by TSH. Patients with partial TSH resistance display a compensated euthyroid condition with high TSH levels, normal thyroid hormone levels, and a normal-sized eutopic thyroid gland. Severe CH is linked with noticeable thyroid hypoplasia, which can be mistaken for athyreosis if TSH resistance is complete. Since early fetal thyroid development and TG synthesis are TSH-independent, serum TG is always detectable in these patients, who display "apparent athyreosis" [21]. The thyroid ultrasound scanning and scintigraphy linked with the perchlorate test and detection of iodine organification defects, however, may not always be able to determine the root cause of TDH. At present, the known variants of DUOX2 gene c.3200C>T, p.S1067L, and TPO gene c.404C>A, p.P135H identified by WES are weekly associated with TDH. Hence, the clinical relevance of looking for the additional mutations with strong associations was obvious using different methods. Quite a few molecular approaches have been developed and put into practice with the idea of generating faster findings and speedy identification of diseases and genetic abnormalities or minor deletions (often exonic sequences). Due to the public availability of human genome sequences, PCR-based sequencing is emerging as a formidable and potent technology. The most effective methods among the various PCR versions used for mutation detection include multiplex PCR and allele-specific (AS)-amplification refractory mutation system (ARMS) - PCR/ AS-ARMS-PCR [30-35]. Out of which, the clinical utility of AS-ARMS- PCR has not been explored in the detection of TSHR gene mutation c.1349G>T, p.arginine[R]450leucine[L] in TDH cases. Additionally, the incidence and prevalence of TSHR gene variant c.1349G>T in the East Asian population is high and most common [36], however, the WES performed using our sample could not detect this mutation. The extensive and well-presented outlining of the differences in genetic variants associated with CH in different racial and geographic locations is of great interest if the findings are relevant to patient care and inform patient-centered care (PCC) practice/evaluation methodologies. Therefore, this study was planned to test the

TSHR gene mutation in an Indian family due to its differential response as a result of altered mutations at c.1349G position (G>A; p.arginine[R]450histidine[H], G>T; p.R450L and G>C: p.arginine[R]450proline[P]) and its key role in the regulation of thyroid hormone biosynthesis and iodide organification. Applying AS-ARMS- PCR for the first time, we report the whole Indian family carrying an autosomal heterozygous variant (SNV-TSHR (NM_000369.5):c.1349 G>T,p.Arg450Leu in the hotspot region of the TSHR gene (https://varsome.com/variant/hg19/TSHR%3Ac.1349G>T?annotation-mode=germline, https://www.ncbi.nlm.nih.gov/snp/rs189261858#clinical significance). The AS-ARMS-PCR creates amplicons of various sizes (containing desired genomic loci) with the help of numerous sets of target-specific primers into a single tube-single PCR reaction mixture. Amplification of the target gene was achieved by precise controlling of the PCR conditions (like annealing temperature, primer concentration, extension time, number of PCR cycles, template concentration, and PCR buffer [37]) and if the nucleotide at the primer's 3′ ends is the same as the bases at the mutated position, with a mismatch being "refractory" to the amplification. When amplifying the normal gene but not genes with the mutant, or vice versa, a PCR result should be produced if the 3′ ends of the primer are thought to be overlapping with the normal gene. The only method for quickly examining known mutations in the genome is by detection of amplified products on agarose gel electrophoresis. Our study also reports the same, where annealing temperature and primer concentration were two of the most important parameters to be adjusted in the AS-ARMS-PCR reaction. The equimolar concentration of the primers was found to be necessary for even amplification of all loci or the desired products. PCR reactions were performed thrice after optimization; the same and reproducible results were noted. The interpersonal and intrapersonal validation of AS-ARMS-PCR was performed and found satisfactory. It cuts down on preparation time, expensive DNA polymerase, and template costs [38]. High-throughput single nucleotide polymorphism (SNP) genotyping and mutation analysis are possible through the use of AS-ARMS-PCR. With AS-ARMS-PCR, false negative results are frequently revealed because each amplified product includes an internal control with an extra amplicon. Thus the method can be adopted for the routine screening of known mutations in large samples.

Pathogenic TSHR gene variants had different amino acid changes in different races. Patients with CH are more likely to have TSHR gene mutations in Japan, and the mutation R450H makes up 70% of the mutant alleles [39]. Recent studies showed that the compound heterozygous TSHR gene mutation c.1349G>A, p.R450H is causing clinically mild CH phenotypes [15], wherein an appropriate increase in TSH serum levels can compensate for the reduced sensitivity of the thyroid (partially or fully compensated TSH resistance) [21]. Lee et al (2011) highlighted that the homozygous mutations in the TSHR gene are known to cause a mild phenotype while a mono-allelic mutation usually causes hypothyroidism with a normal phenotype [40]. Similarly, four TSHR gene mutations c.1349G>A, c.1207G>A, c.394G>A, and c.611C>T in the CH patients affecting the TSH-induced cAMP production as well as TSH

receptor binding activity including milder effect due to c.1349G>A, p.R450H have been reported in Japanese [20]. Mutation in the TSHR gene causes a spectrum of phenotypes with severity correlating with the number of mutated TSHR alleles and varied degrees of receptor functional impairment [41]. The R450H mutant showed somewhat reduced cell surface expression and slightly worsened receptor functioning [42]. Only one report of DUOX2 gene mutation in the Indian subcontinent in infants with severe *in-utero* deficiency of TH and a very short duration of hypothyroidism has been reported [43]. Balmiki et al., (2014) reported the association of the TPO gene [rs732609 (Thr725Pro) and rs1126797 (Asp666Asp)] with hypothyroidism in India [44]. However, the collective effect of mutations in the DUOX2, TPO, and TSHR genes was not reported. Several studies noticed that a defect in the system that generates H_2O_2 causes permanent CH and necessitates lifelong TH replacement [45]. Consanguineous and non-consanguineous families with CH have shown high levels of mutations in TPO and DUOX2 genes [46-48]. Moreover, the disease-associated variants of both genes varied geographically. The majority of the mutations have been reported in DUOX2 genes, the variant p.S1067L was higher in cases than those in the controls (0.286 versus 0.085, OR = 4.306, P = 0 001) and reported as a likely disease-causing mutation in HGMD [49,50]. A recent study reported most of the variants in TPO (68.2%) and DUOX2 (27.3%) genes [51]. A recent study by Wang et al., (2021) classified TPO gene variant c.404C>A, p.Pro135His as variants of uncertain significance [52]. The substitutions mutations, namely, p.Ala373Ser, p.Ser398Thr, and p.Thr725Pro, had been involved in Bangladeshi patients with TDH and the molecular docking-based study revealed that these mutations had a damaging effect on the TPO protein activity [53].

Our study expanded the spectrum of biological processes (GO) enrichment analysis using 409 variants of unknown categories identified via next-generation sequencing (NGS) pointing towards the detection of stimulus (GO:0051606) and hyperphosphatemic tumoral calcinosis (HFTC*)* due to defective N-acetylgalactosaminyltransferase-3 (GALNT3; R-HAS-5083625**; Figure 5)**. However, this observation does not significantly match the disease phenotype. Our study also stretched the spectrum of mutation analysis in the DUOX2, TPO, and TSHR genes identified via NGS. Out of 131 different variants identified by WES in the TDH child, missense mutations in DUOX2 gene c.3200C>T, p.S1067L, TPO gene c.404C>A, p.P135H, and TSHR gene c.2181G>C, p.glutamic acid (E)727aspartic acid (D) (rs1991517) were found to be clinically associated with TDH. The known TSHR gene variant c.2181G>C, p.E727D lies within the intracellular portion of the receptor and was identified by our study. This is consistent with the results of the earlier study by Kollati et al [54], which showed that the presence of the G-allele raised the risk of CH by 41% (OR: 1.41, 95% CI 1.03-1.93) and 45% (OR: 1.45, 95% CI 1.20-1.76) in fixed effect and random effect models, respectively. In the fixed-effect model, the risk of CH is increased by 2.3-fold (95% CI 1.32-3.99) for the GG- genotype. The rs1991517 mutation (c.2181GAG>GAC, p.Asp727Glu) in the TSHR gene was reported to alter the binding affinity to cAMP (ΔG of 727D vs.727E:−7.27 vs.−7.34 kcal/mol) [54].

Consequently results in altering cAMP-mediated signal transduction and is reported to increase the genetic risk for CH. Ho et al., 2003 reported significant ethnic differences in the distribution of TSHR gene polymorphisms for D727E mutations. It is more common among the Chinese than Indians and Malays ($p<0.0001$) [55]. Similarly, double heterozygosity was substantially more common in CH patients in Japan than in the general population [22]. A recent study showed double heterozygosity for likely pathogenic variants of the TSHR gene and the DUOX2 gene and concluded that genotype alone does not appear to determine clinical follow-up and the need for replacement therapy [56]. The various factors that affect the function of the TSHR gene or changes in TH needed with aging can explain the different phenotypes observed in heterozygous carriers of TSHR gene mutations. Unfortunately, it can be quite challenging to monitor subclinical or mild hypothyroidism patients over time and opinions differ on whether replacement therapy is necessary for such cases. However, these patients are at risk for neurodevelopmental issues, if left untreated. These patients may also develop overt hypothyroidism during puberty or pregnancy where higher levels of thyroid hormone are required. This may be the case observed in the subject (mother) of our study where she has developed gestational hypothyroidism. Thus it is crucial to conduct regular clinical and biochemical follow-ups to monitor the health of heterozygous carriers of TSHR gene mutations such as c.1349G>T, p.R450L. Maruo et al (2008) believe that complete inactivation of DUOX2 causes transient CH but not permanent CH for the following reasons. 1) An additional H_2O_2-generating oxidase exists in thyrocytes, namely DUOX1, although the exact role of DUOX1 in TH synthesis has not been determined. The amount of DUOX1 expressed is one-fifth that of DUOX2. Even if the enzyme activity of DUOX2 is lost, a low level of H_2O_2 supply may be maintained by DUOX1. Neonates with transient CH caused by TDH due to genetic defects show loss of DUOX2 activity, subclinical reduction of hormone synthesis gradually with age, and development of adult-onset hypothyroidism with senescence [45]. After the improvement in the serum hormone level, there is still a risk of hypothyroidism recurring when there is an increased requirement for TH during conditions like pregnancy. Identification of genetic causes of transient CH in patients with no obvious external symptoms of hormone defect is therefore important in maintaining their subsequent health.

Limitations

Our study has some limitations, as it is a single family-based study. The identified mutations need to be confirmed by Sanger sequencing. A heterozygous sample's genetic typing may be inaccurate or unclear as a result of the preferential PCR amplification of one allele compared to another. Such preferential PCR amplification may be caused in several ways. First, if the reaction's conditions—such as the denaturation temperature (T’den), the time spent at the T'den, the concentrations of salt and co-solvent, etc.—allow one allele to denaturation but not the other, and preferential amplification may result from significant GC% differences between alleles. When two alleles in a heterozygous sample are represented by amplification products that denature at different

temperatures (differential denaturation) may result in preferential amplification. An inaccurate genetic typing of such material could occur when the allele denatures less effectively and goes undetected. Preferential amplification can also result from differential stimulation of an allele's synthesis. A well-known PCR technique is allele-specific amplification, which is based on the ineffective extension of oligonucleotide primers that are mismatched with the template at the 3' end [34, 57]. When starting a reaction from a heterozygous sample with a very small number of target DNA molecules, preferential amplification due to stochastic fluctuation (sampling error) can happen; the likelihood of an unequal sampling of the two alleles of a heterozygote is increased when doing so (unless the DNA is amplified directly from un-extracted diploid cells). By changing the cycle number so that at least 20 copies of the target DNA are needed to get a typing result for that PCR machine, this issue can be avoided. It would be expected that any major discrepancies in amplification efficiency between a heterozygote's two alleles would lead to unequal dot intensities for the two alleles when the cycle number was raised. The findings of this study cannot be generalized universally or to a large population unless the findings are reported in a series of patients and subjected to some form of statistical analysis. Though both AS-ARMS–PCR, and WES are used often in routine diagnosis, and many a time requires optimization for reproducible results. We expect that a detailed description of the methodology along with scientific findings can help the researchers perform the same in their labs, more specifically the beginners. This study also helps the readers and pediatric critical care providers to think beyond their knowledge and make use of advanced techniques. In conclusion, the coexistence, severity, and expressivity of multiple pathogenic mutations at multiple sites or multiple genes, the sum effect of rare alleles, monoallelic/ monogenic, biallelic/ digenic, oligogenic or polygenes, and exonic variants may contribute to more serious and heterogeneous CH pattern, clinical expression, cross-loss of enzyme activity, early and permanent onsets [16, 58]. Moreover, pathogenic TSHR gene variants had different amino acid changes in different races and may contribute to different clinical presentations in different populations. Our study reports that the known variants of DUOX2 gene c.3200C>T, p.S1067L, and TPO gene c.404C>A, p.P135H are clinically associated with TDH. The TSHR gene variant c.1349G>T, p.Arg450Leu (an autosomal heterozygous), and other mutations (e.g. c.3200C>T, and c.404C>A) might contribute to clinically more severe TDH in children born to carrier parents and may be associated with TSH resistance. Mutations in the TSHR gene may be considered one of the causes of TDH in Indian patients [59] and the AS-ARMS-PCR can be a method of choice for screening known genetic variants in a large-scale population. We recommend a large-scale family-based genetic investigation and confirmation of the variants by Sanger sequencing. The identified variants need to be established in multiple families (at least ≥ 3) with positive (diseased) and negative (healthy) controls for their pathogenic associations with clinical presentations along with their functional relevance**.**

Highlights

- WES analysis of the genes known to cause TDH showed variants of the DUOX2 gene [c.3200C>T, p.Ser(S)1067L] and TPO gene [c.404C>A, p.Pro(P)135His(H)], and TSHR gene variant c.1349G>T/p.R450L by AS-ARMS-PCR.
- Out of 131 variants identified in TDH-causing genes by WES, DUOX2 gene variant c.3200C>T, p.S1067L and TPO gene variant c.404C>A, p.P135H were noticed to be clinically associated with TDH. Applying AS-ARMS-PCR for the first time, we report the whole Indian family carrying an autosomal heterozygous variant (c.1349G>T/p.R450L) in the hotspot mutation region of the TSHR gene.
- The known TSHR gene variant c.1349G>T/p.R450L (an autosomal heterozygous), and other variants of the DUOX2 gene (c.3200C>T, p.S1067L) and TPO gene (c.404C>A, p.P135H) might contribute to clinically more severe TDH in children born to carrier parents.

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Author's contributions

Dr. Venkanna Bhanothu: conceptualization, designing of methodology, writing-original draft, and supervision; Simeone Fernandes: performed the experiments. Dr. Sudha Chandrashekhar Rao, Dr. Rachna Keshwani, and Dr. Shagun W have performed the clinical evaluation. Dr. Suchitra Surve, Dr. Shailesh Pande, Dr. Sudhakar DVS and Mrs. Neha Minde have reviewed the manuscript. Mr. Shivamkumar Sriwas has repeated some experiments.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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Supplementary materials

Determining the concentration of PCR product mathematically

We need 2-5 ng of DNA to be recognized as a band on agarose gel electrophoresis = 5 * 10^{-6} grams. In this case, we have a PCR product of internal control = 654 bp. Then, the molecular weight of 654 bp = 654 * 650 = 425100 Daltons. Number of moles = weight / molecular weight = 5 * 10^{-6} / 425100 = 1.1762 * 10^{-11}. Number of molecules = number of moles * Avogadro number = 1.1762 * 10^{-11} * 6.02214 * 10^{23} mol^{-1} = 7.083 * 10^{12}.

Determining optimum PCR product concentration as per the following equation:

The PCR product copy number = started copy number of the target DNA * $2^{\text{number of PCR cycles}}$ 7.083 * 10^{12} = started copy number of the target DNA * 2^{35} (i.e., 10^{11} copies after 35 cycles). The started copy number of the target DNA = 7.083 * 10^{12} / 10^{11} = 70.83. However, 10^4 to 10^5 copies of the target DNA to the final volume of the PCR reaction are generally recommended.

Supplementary Tables:

Supplementary **Table S1:** Components of AS-ARMS-PCR reaction for TSHR gene amplification (total volume for 25 µl)

Reaction components with an initial Concentration		All sets of primers (in µl)	T-allele primer (in µl)	G-allele primer (in µl)	Internal control (in µl)	Final concentration
Milli Q		16.625	18.25	19.625	19.125	-
10X Taq Buffer		2.5	2.5	2.5	2.5	1X
dNTP mix (10mM)		1	1	1	1	400 µM
Taq DNA Polymerase (5U)		0.25	0.25	0.25	0.25	0.05 U
Template genomic DNA (19.91ng/µl of TDH child)		0.5	0.5	0.5	0.5	0.4 ng/µl
Primers (10 pmol/µl)	OFP	1.5	1.5	-	1.5	0.6 pmol/µl
	IFP	1	-	1	-	0.4 pmol/µl
	IRP	1	1	-	-	0.4 pmol/µl
	ORP	0.625	-	0.625	0.625	0.25 pmol/µl

Supplementary **Table S2:** Optimized reaction components of two looped touchdown-AS-ARMS-PCR with 2x Superplex master mix (total volume 25 µl)

Reaction components		**TDH Child's genomic DNA (19.91 ng/µl) in µl**	**Mother's genomic DNA (43.56 ng/µl) in µl**	**Father's genomic DNA (46.66 ng/µl) in µl**
Milli Q		7.33	8	8
2x Superplex Mastermix		12.5	12.5	12.5
Template Genomic DNA		1.17	0.5	0.5
1.5 x 10^{12} molecules of each primer (10 pmol/µl)	OFP	1	1	1
	IFP	1	1	1
	IRP	1	1	1
	ORP	1	1	1

Supplementary **Table S3:** Variant prioritization criteria and the number of prioritized variants of WES

Filtering criteria	No. of variants found in CH1C
Total number of variants	60121
Variants after filtering Allele Freq Global Minor < 0.05	12731
Variants within ± 20 bp on both sides of exon boundaries	7473
Read depth > 20	6475
Sift (deleterious and blank) excluding tolerated	5644
PolyPhen (all damaging, unknown, and blanks) excluding benign	5455
CADD Score >=20 and NA	631
Consequence; missense_variant, frameshift_variant, frameshift_variant,splice_region_variant, stop_gained, protein_altering_variant, frameshift_variant,start_lost, stop_gained,frameshift_variant, stop_gained, inframe_deletion, frameshift_variant,stop_lost, splice_donor_variant, stop_gained,splice_region_variant, stop_gained,inframe_deletion, frameshift_variant,stop_retained_variant, missense_variant,splice_region_variant, inframe_insertion, splice_acceptor_variant and blank excluding intronic and noncoding variants	461
Variants with transcripts	409

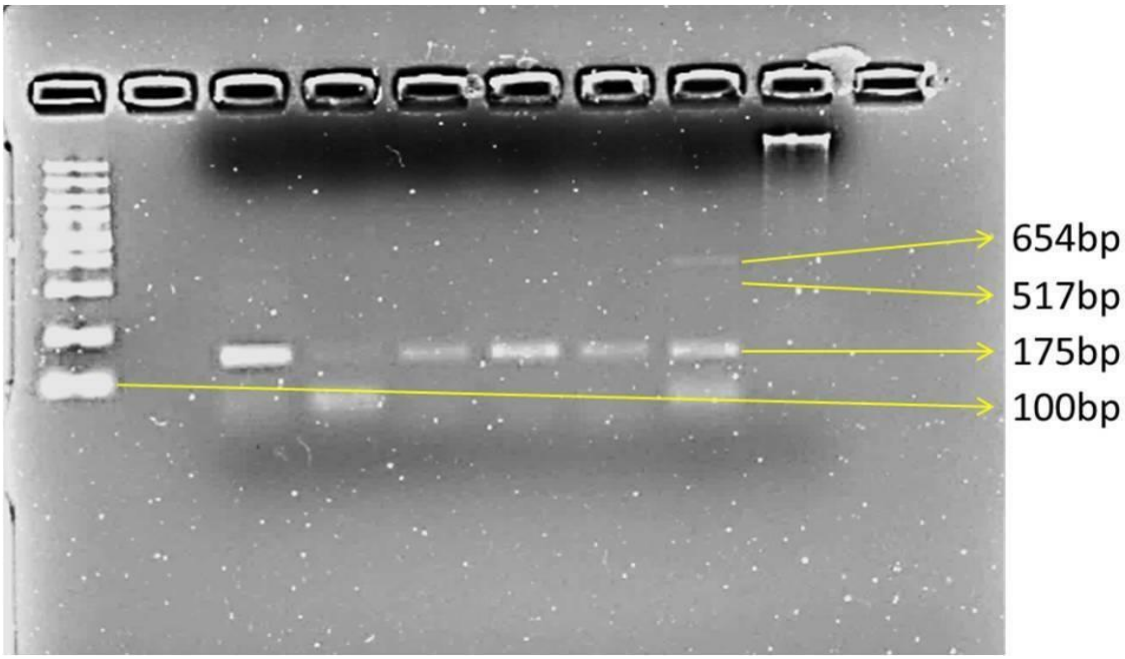
Supplementary Table S4: Summary of biological interpretation using PPI Network & MCODE Components

Network	Annotation of CH1C
MyList	R-HSA-5083636|Defective GALNT12 causes CRCS1|-9.9;R-HSA-5083625|Defective GALNT3 causes HFTC|-9.9;R-HSA-5083632|Defective C1GALT1C1 causes TNPS|-9.7
MyList_MCODE_ALL	R-HSA-913709|O-linked glycosylation of mucins|-14.5;R-HSA-5083625|Defective GALNT3 causes HFTC|-13.9;R-HSA-5083636|Defective GALNT12 causes CRCS1|-13.9
MyList_SUB1_MCODE_1	R-HSA-913709|O-linked glycosylation of mucins|-21.7;R-HSA-5173105|O-linked glycosylation|-19.6;R-HSA-5083636|Defective GALNT12 causes CRCS1|-18.5
MyList_SUB1_MCODE_2	hsa05330|Allograft rejection|-17.6;hsa05332|Graft-versus-host disease|-17.3;hsa04940|Type I diabetes mellitus|-17.2

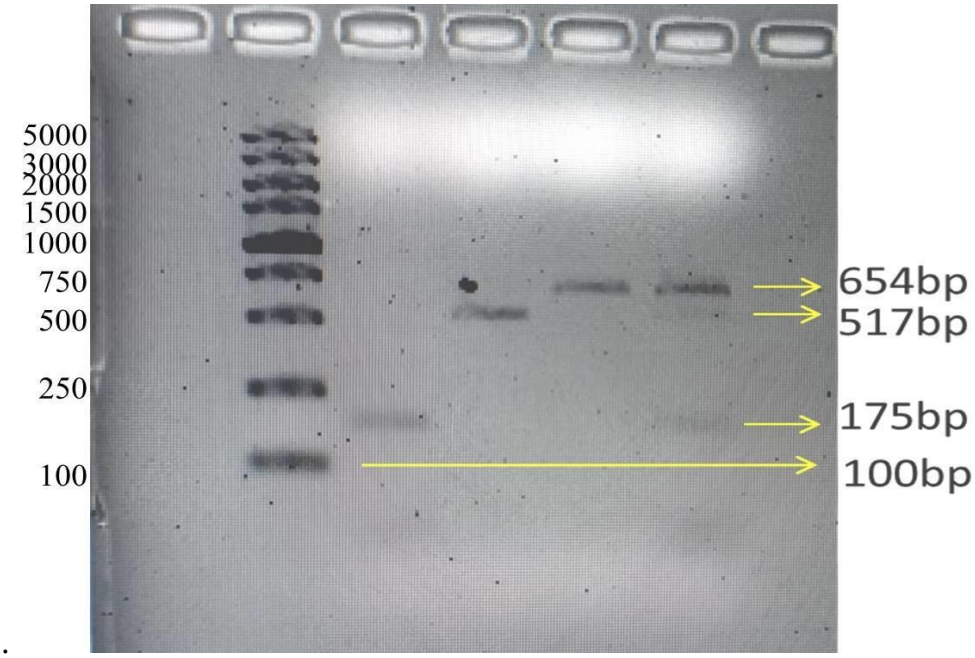
MyList_SUB1_MCODE_3	GO:0031175|neuron projection development|-5.5;GO:0007411|axon guidance|-5.1;GO:0097485|neuron projection guidance|-5.1
MyList_SUB1_MCODE_5	M3005|NABA COLLAGENS|-8.5;R-HSA-8948216|Collagen chain trimerization|-8.5;R-HSA-1650814|Collagen biosynthesis and modifying enzymes|-8.0
MyList_SUB1_MCODE_6	M47|PID INTEGRIN CS PATHWAY|-9.2;GO:0033627|cell adhesion mediated by integrin|-8.7;WP2118|Arrhythmogenic right ventricular cardiomyopathy|-7.8
MyList_SUB3_MCODE_7	R-HSA-418594|G alpha (i) signalling events|-5.9;hsa04080|Neuroactive ligand-receptor interaction|-5.8;R-HSA-500792|GPCR ligand binding|-5.4

Supplementary Figure

Supplementary Figure S1: Primer quality and intactness on 2% agarose gel electrophoresis. Details of the loading pattern are given in the figure. The molecular weights of the DNA ladder start with 100 base pairs (bp) from the bottom (250bp, 500bp, and 750bp) and end with 5000bp on the top.



Supplementary Figure S2: The AS-ARMS-PCR amplified products using all sets of primers and template genomic DNA of a child with TDH on the 1.5% agarose gel electrophoresis. Well 1- DNA Ladder (100bp [from bottom], 250bp, 500bp, 750bp, and 5000bp [on the top]), Well 3- annealing temp 50^{o}C, Well 4- annealing temp 52^{o}C, Well 5- annealing temp 54^{o}C, Well 6-annealing temp 56^{o}C, Well 7- annealing temp 58^{o}C, Well 8- annealing temp 60^{o}C, Well 9- Genomic DNA



Supplementary Figure S3: The AS-ARMS-PCR amplified products at 62^{o}C annealing temperature on the 1.5% agarose gel electrophoresis. Well 1- empty, Well 2- DNA Ladder, Well 3- T-allele (175 bp)

product, Well 4- G- allele (517 bp) product, Well 5- Internal control (654 bp) product, Well 6- all three bands were observed using all sets of primers in a single tube –single reaction.

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