Molecular Biology Reports
https://doi.org/10.1007/s11033-020-05803-x

ORIGINAL ARTICLE

# Newborn screening and single nucleotide variation profiling of *TSHR*, *TPO*, *TG* and *DUOX2* candidate genes for congenital hypothyroidism

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Received: 20 June 2020 / Accepted: 3 September 2020


## Abstract

High prevalence of congenital hypothyroidism (CH) among Indian newborns prompted us to establish population-specific reference ranges of TSH and to explore the contribution of the common genetic variants in *TSHR*, *TPO*, *TG* and *DUOX2* genes towards CH. A total of 1144 newborns (593 males and 551 females) were screened for CH. SNV profiling (n = 22) spanning three candidate genes, i.e. *TSHR*, *TPO* and *TG* was carried out in confirmed CH cases (n = 45). In screen negative cases (n = 700), ten *TSHR* variants were explored to establish association with CH. No mutation found in *DUOX2*. The 2.5th to 97.5th percentiles of TSH in these newborns were 0.5 to 12.2 mU/L. In newborns with optimal birth weight, the cut-off was 10 mU/L. Lower or higher birth weight resulted in slightly higher TSH. Two *TSHR* variants, i.e. rs7144481 and rs17630128 were associated with agenesis, hypoplasia and goiter. The rs2268477 was associated with agenesis and hypoplasia. The rs1991517, rs2075176 and rs2241119 were associated with agenesis only. The rs7144481, rs17630128, rs1991517 and rs2268477 were associated with 2.17, 4.62, 2.91 and 2.29-fold increased risk for CH, respectively. Among the *TPO* variants, rs867983 and rs2175977 were associated with agenesis and goiter, respectively. Among the *TG* variants, rs2076740 showed association with agenesis and goiter. Two rare variants i.e. *TPO* g.IVS14-19 G>C and *TG* c.1262 C>T were observed in CH cases. No genetic variant identified in the two exons of *DUOX2*. To conclude, the current study established Indian population-specific normative values for TSH and demonstrates specific genotype–phenotype correlations among three candidate genes.

**Keywords** Congenital hypothyroidism · *TSHR* · *TPO* · *TG* · *DUOX2* · Newborn screening

## Introduction

Congenital hypothyroidism (CH) is one of the most prevalent metabolic disorders in the newborn with a prevalence of 1:4000 globally [1, 2] and 1:1100 in India [3]. CH is classified into permanent CH and transient CH. The permanent CH can be subdivided into primary CH (PCH) and

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**Electronic supplementary material** The online version of this article (https://doi.org/10.1007/s11033-020-05803-x) contains supplementary material, which is available to authorized users.

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Published online: 15 September 2020

secondary CH (SCH) [4, 5]. PCH is further classified into thyroid dysgenesis and thyroid dyshormonogenesis [4, 6]. The majority of these cases (80–85%) have defects in thyroid gland development known as thyroid dysgenesis [7]. Thyroid dysgenesis is ranging from the lack of thyroid gland (agenesis; 35–40%), to located in sublingual position (ectopic; 55–60%) or small thyroid gland or remnants thyroid gland in the normal position (hypoplasia; 5%) [8]. Remaining 15–20% cases are caused by defects in the thyroid hormone biosynthesis known as dyshormonogenesis [8]. Defects in various genes such as a thyroid stimulating hormone receptor (*TSHR*), paired box 8 (*PAX8*), thyroid transcription factor 1 (*TTF-1*) and thyroid transcription factor 2 (*TTF-2*) were reported to have association with thyroid dysgenesis [9] while defects in thyroid peroxidase (*TPO*), thyroglobulin (*TG*), solute carrier family 5 member 5 transporter (*SLC5A5,* encoding *NIS*), solute carrier family 26 member 4 transporter (*SLC26A4* encoding *PDS)*, Dual oxidase (*DUOX1* and *DUOX2*), Dual oxidase maturation factors (*DUOXA1* and *DUOXA2*), and iodotyrosine dehalogenase 1 (*DEHAL1*) were reported to have association with dyshormonogenesis [7].

Thyroid stimulating hormone (TSH) binds to *TSHR* and prompts the release of two signal transduction pathways: G-alpha (s) ($G_s$) and $G_q$ proteins followed by activation of adenylate cyclase-cyclic adenosine monophosphate (cAMP) and phospholipase C (PLC)-calcium cascade, respectively [10, 11]. These pathways are involved *TG* iodination and cell proliferation. The pathway of Gs is responsible for thyrocytes iodine uptake regulation [11]. The $G_q$-phospholipase C-calcium cascade activates the *DUOX2* protein function [10]. TSH and its receptors play an important role in the maturation and differentiation of thyroid development in the late stage [12]. The *TPO* is involved in catalyzing iodide oxidation, iodination of tyrosine residues and coupling of mono- and di-iodotyrosyl in *TG* molecule to synthesize triiodothyronine ($T_3$) and thyroxine ($T_4$) [13]. This activity needs the presence of hydrogen peroxide ($H_2O_2$) which is generated by *DUOX2* [7].

## Materials and methods

### Recruitment of subjects

This project is an offshoot of the newborn screening (NBS) program that is being carried out by the lead investigator from 2001 to till date where in 49,432 newborns were screened [3, 14], out of which 45 CH cases were diagnosed (incidence: 1:1098). From this cohort, 1099 screen negative cases were randomly selected along with the 45 confirmed cases of CH (593 males and 551 females) (Fig. 1).

In the newborn screening, TSH levels were measured using a commercial neonatal TSH kit (Perkin Elmer GSP neonatal kit). The diagnosis of CH was confirmed if the infant has a low $T_4$ (< 10 µg/dL) and TSH > 40 mU/L. The biochemically diagnosed cases were subjected to other optional tests, including thyroid ultrasonography, thyroid uptake and scan. Presence of an ectopic gland was suggestive of permanent congenital hypothyroidism. Absence of thyroid gland uptake was suggestive of thyroid aplasia or hypoplasia. Normal scan or a goiter was suggestive of a genetic defect in $T_4$ synthesis. All the diagnosed cases of CH were further evaluated genetically using the candidate genes, i.e. *TSHR*, *TPO*, *TG* and *DUOX2*. A total of 700 screen negative cases was evaluated genetically for *TSHR* variants. The study protocol was approved by the institutional Ethical committee of Rainbow Children's Hospital (RCHBH/066/02-2018), Hyderabad. Informed consent was obtained from the parents or guardians of the enrolled neonates. The exclusion criteria were associated congenital malformations, Down's syndrome and Transient neonatal hyperthyrotropinemia.

### DNA isolation and amplification

The DNA was isolated from buffy coat peripheral white blood cells by QIAamp blood kit (QIAGEN, Hilden, Germany). Quality and quantity of isolated DNA were analyzed by using a NanoDrop™ 1000 (Thermo Fisher Scientific, Waltham, USA).

Gene Tool 1.0 [15] computer program was used for primer designing. As shown in the Supplementary Table 1, the specific primers were used in amplifying *TSHR*, *TPO*, *TG* and *DUOX2* gene exons and their exon–intron boundaries. Primers for the amplification of exon 8 of the *TPO* gene were synthesized based on the previous research report [16].

As shown in the Supplementary Table 1, the exonic regions of the *TSHR*, *TPO*, *TG* and *DUOX2* genes, including the splicing regions, were amplified by PCR. The PCR reaction mixture contained 50–100 ng of genomic DNA, 10 pmol of each forward and reverse primers, 1.5 mM $MgCl_2$, 10 mM of each dNTP, 5 µL 10 × PCR buffer, 5U Taq DNA polymerase (Thermo Fisher Scientific, Waltham, USA) at a final volume of 50 µL as in other reports [17]. The conditions for PCR amplification were an initial denaturation of 5 min at 95 °C, followed by 30 cycles of amplification consisting of denaturation at 95 °C for 30 s, annealing at 51–65 °C (based on suitable annealing temperature for each of the primer) for 30 s and extension at 72 °C for 30–60 s and with a final extension at 72 °C for 5 min. The amplified PCR products were analyzed in 2% agarose gel and followed by purified with ExoSAP-IT™ PCR Product purification kit (Applied Biosystems, Foster City, USA) as described in our previous report [18].

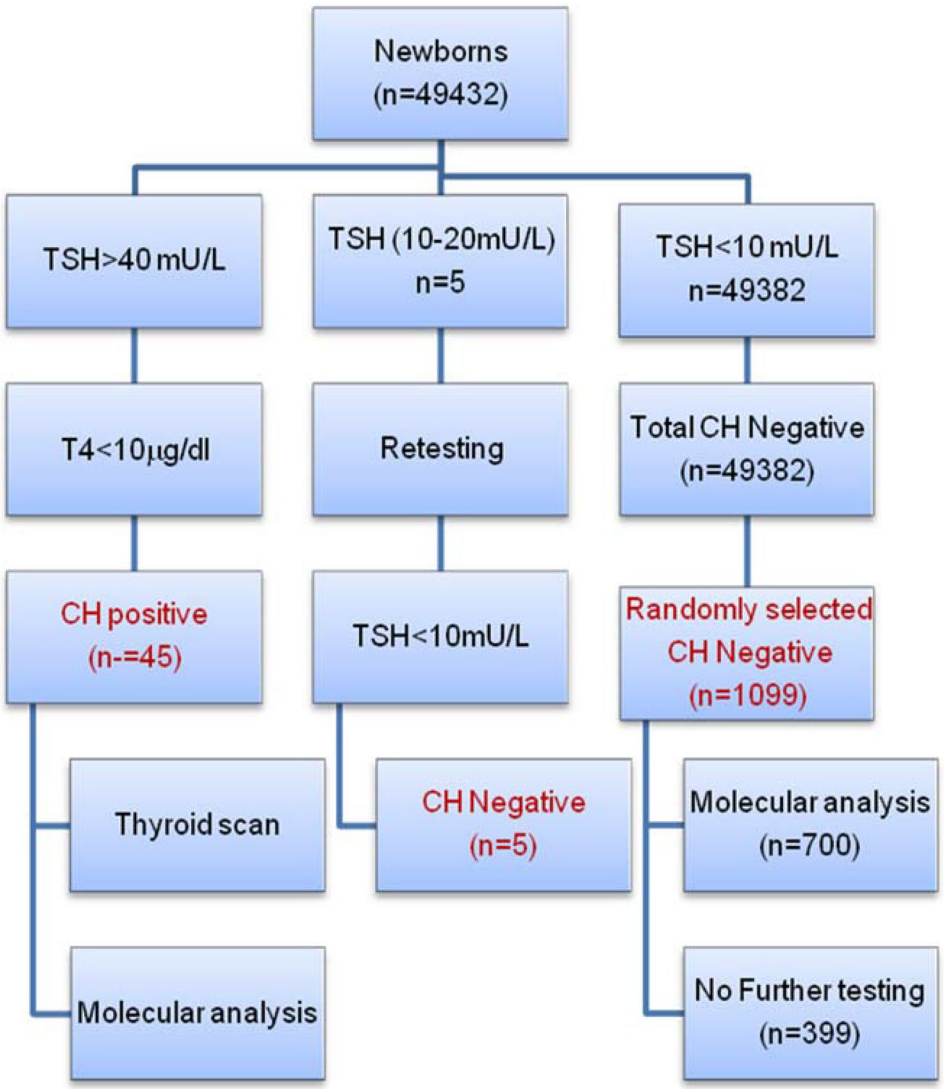


**Fig. 1** Process flow from newborn screening to molecular testing. This illustrates the process of various stages of testing and the respective sample size at each stage

## DNA sequencing and data analysis

The DNA sequencing was performed with ABI 3730xLDNA Analyser (Applied Biosystems, Foster City, USA). The sequence variants nomenclature refers to the NCBI human *TSHR*, *TPO*, *TG* and *DUOX2* reference nucleotide sequence (NCBI accession number NM_000369, NM_000547, NM_003235 and NM_014080, respectively). The 'A' of the ATG of the start codon denoted as nucleotide + 1 and the start codon methionine is denoted as codon 1.

Sequence chromatogram data results were analyzed using Chromas 2.6.2 and BLAST (https://www.ncbi.nlm.nih.gov/blast/) software programs. Nucleotide alterations were compared with the 1000 Genomes (https://www.1000genomes.org/) and dbSNP (https://www.ncbi.nlm.nih.gov/snp/) databases as described in our previous report [18].

## Sequence variants frequencies and prediction of functional effects

The minor allele frequencies (MAF) of genetic variants were computed from a genome aggregation database (gnomAD; http:// https://gnomad.broadinstitute.org/) and 1000 Genomes Projects (https://www.internationalgenome.org) based on their rs IDs.

The functional effects of the genetic variants were explored using three different in silico tools: SIFT (https://sift-dna.org), PolyPhen-2 (https://genetics.bwh.harvard.edu/pph2/) and PROVEAN (https://provean.jcvi.org.) were used.

## Statistical analysis

Fisher exact test was used to establish genetic associations where in 2 × 2 contingency table was computed based on the presence or absence of genetic variantion in the presence

or absence of a particular phenotype. In view of multiple genetic variants, the data representation was restricted to minor allele frequencies and p values. The odds ratios and 95% confidence intervals were not depicted. Student t-test and analysis of variance were used for exploring the differences in mean ± SD among two or more groups, respectively. Heat-map analysis was carried out to demonstrate the genotype–phenotype associations with respect to thyroid scan results.

## Results

### Establishment of reference intervals for TSH through newborn screening data

In screen negative newborns, the 2.5th to 97.5th percentiles of TSH in this cohort were 0.5–12.2 mU/L. The data was segregated based on gender (male and female) and birth weight (< 2.0 kg, 2.0–3.5 kg and > 3.5 kg) to explore physiological variations in TSH. It was observed that neonates with lower (male: 0.30–12.0 mU/L; female: 0.92–10.36 mU/L) or higher (male: 0.55–12.34 mU/L; female: 0.5–12.24 mU/L) birth weight exhibit a slight increase in TSH levels compared to those with optimal weight (male: 0.60–10.23 mU/L; female: 0.40–9.90 mU/L) (Fig. 2). However, this increase is not statistically significant.

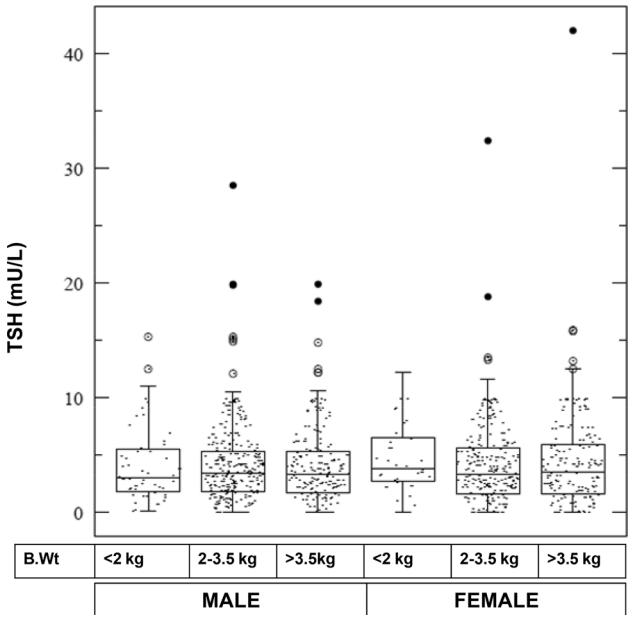


**Fig. 2** Distribution of TSH levels based on gender and birth weight. In both genders, newborns with optimal birth weight showed a TSH cut-off of 10 mU/L while newborns with lower and higher birth weight had a cut-off higher by 2 mU/L

### Thyroid scan in confirmed CH cases

Out of 45 confirmed CH cases, 35 opted for thyroid scan and the results are as follows: agenesis (n = 7), hypoplasia (n = 5), goiter (n = 7), no significant abnormalities (n = 16). Cases with goiter showed maximum increase in TSH (206.71 ± 119.28 mU/L) and lowest $T_4$ (1.18 ± 0.73 µg/dL). In cases with hypoplasia, TSH levels were 156.06 ± 94.18 mU/L and $T_4$ levels were 1.45 ± 0.92 µg/dL. In thyroid agenesis, TSH levels were the lowest (119.4 ± 99.49 mU/L) and $T_4$ levels were maximum (2.89 ± 2.39 µg/dL) (Fig. 3; Supplementary Table 2).

### Genetic analysis

A total of 22 genetic variants was tested in three candidate genes: *TSHR* (n = 10), *TPO* (n = 8), *TG* (n = 4). The MAFs of cases with no significant thyroid gland anomalies were considered as base line to compare the MAFs of cases with agenesis, hypoplasia and goiter (Fig. 4).

As shown in the Supplementary Table 3, novel variants alleles were found to be very rare on the basis of control data of the datasets are shown. MAFs of Asian, global and total populations were computed based on the datasets of gnomAD and 1000 Genome projects. Out of 22 sequence variants, two missense variants, i.e. rs2175977 and rs2069548 were predicted to be deleterious. Remaining variants were benign.

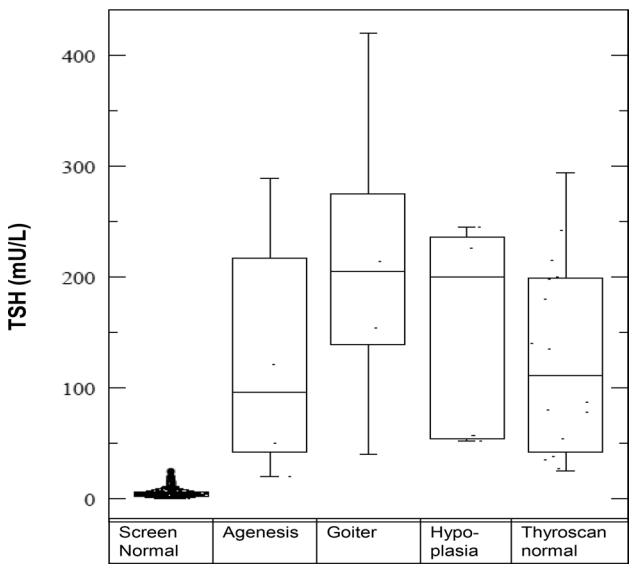


**Fig. 3** Distribution of TSH levels among screen negative and confirmed CH cases. The screen normal newborns have TSH values < 20 mU/L. Goiter and hypoplasia were associated with higher TSH levels than agenesis and normal thyroscan

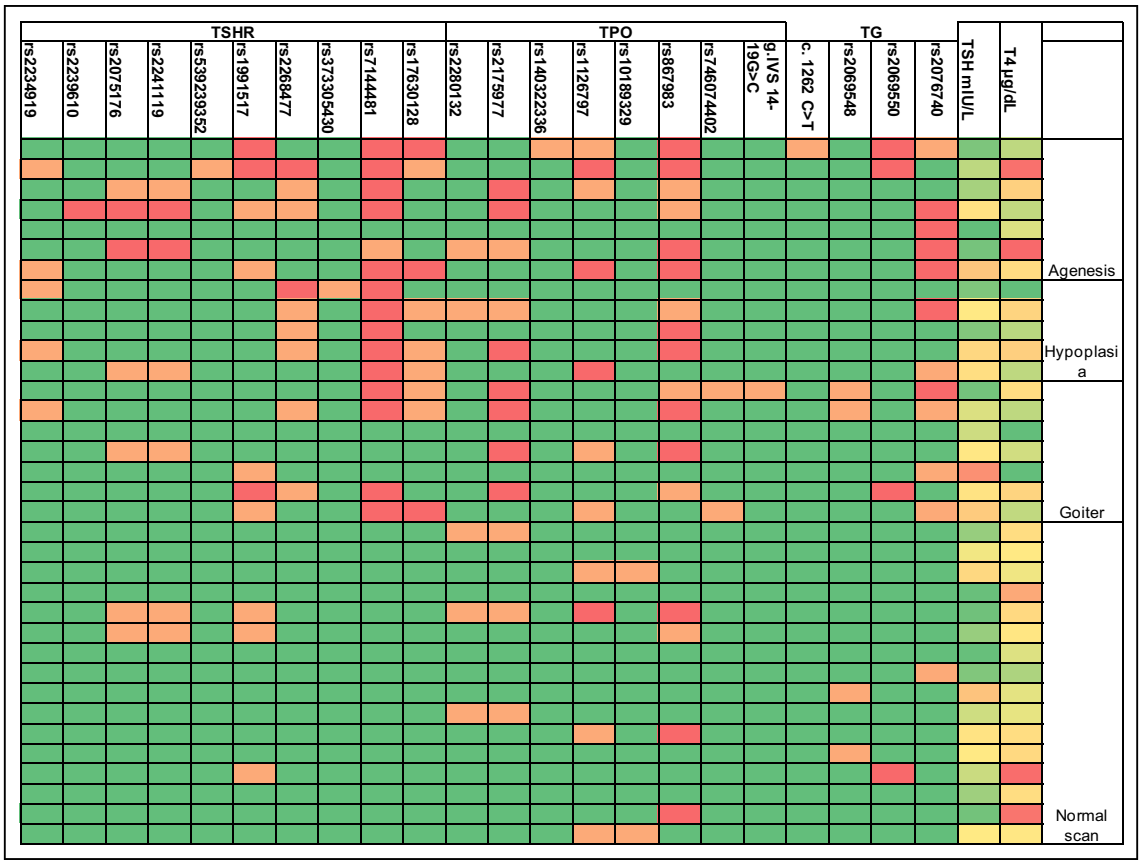


**Fig. 4** Heat map showing the distribution of 22 genetic variants across three candidate genes in different CH phenotypes. The wild, heterozygous and homozygous mutant genotypes were represented as green, yellow and red colors, respectively. The TSH and $T_4$ values were graded according to the levels from green (lowest) to red (highest)

## *TSHR* variants

The rs7144481 variant was absent in cases with normal thyroid scan while its MAFs were higher in cases with abnormal thyroid scan, i.e. agenesis (78.57%), goiter (57.14%) and hypoplasia (100.0%) suggesting a strong association of this variant with CH ($p < 0.0001$). The rs17630128 variant was absent in cases with normal thyroid scan while exhibiting higher MAFs in agenesis (MAF: 35.71%, $p = 0.003$), hypoplasia (MAF: 30.0%, $p = 0.02$) and goiter (MAF: 28.57%, $p = 0.01$). The rs2268477 was absent in cases with normal thyroid scan, but had higher MAFs in hypoplasia (MAF: 50.0%, $p = 0.001$) and agenesis (MAF: 28.57%, $p = 0.01$). However, this variant showed no association with goiter ($p = 0.18$). The rs1991517 variant was associated with agenesis (MAF: 42.86% vs. 9.38%, $p = 0.03$) while showing null association with goiter and hypoplasia. The rs2075176 and rs2241119 variants showed borderline association with agenesis ($p = 0.04$) (Table 1).

We have performed *TSHR* sequencing in 700 screening negative newborns to establish baseline frequencies of the investigated variants and to explore the association with CH in Toto. The rs7144481 variant (Cases vs. controls MAFs: 34.44% vs. 19.50%) showed 2.17-fold (95% CI 1.38–3.42) increased risk for CH. The rs17630128 (MAFs: 12.22% vs. 2.93%) was associated with 4.62-fold increased risk for CH (95% CI 2.29–9.32). The rs1991517 (MAFs: 21.11% vs. 8.43%) variant was associated with 2.91-fold increased risk for CH (95% CI 1.69–4.99). The rs2268477 variant (MAFs 13.33% vs. 6.29%) was associated with 2.29-fold (95% CI 1.20–4.37) increased risk for CH (Table 2).

## *TPO* variants

Among the eight *TPO* variants analyzed, only two showed statistically significant association with CH. The rs2175977 showed association with goiter (MAF: 57.14% vs. 9.38%, $p = 0.002$). The rs867983 variant showed association with agenesis (MAF: 71.43% vs. 21.88%, $p = 0.004$) (Table 1).

## *TG* variants

Among the four variants of *TG* analyzed, only rs2076740 showed association with agenesis (MAF: 64.29% vs. 3.13%,

**Table 1** Association of genetic variants based on CH phenotypes

| Genetic variants | Agenesis | Goiter | Hypoplasia | Normal | Agenesis | | Goiter | | Hypoplasia | | Normal | | Agenesis | Goiter | Hypoplasia |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MAF | MAF | MAF | MAF | M | W | M | W | M | W | M | W | p | p | p |
| *TSHR* variants | | | | | | | | | | | | | | | |
| rs2234919 (c.310 C>A) | 14.29 | 7.14 | 20.00 | 0.00 | 2 | 12 | 1 | 13 | 2 | 8 | 0 | 32 | 0.18 | 0.61 | 0.11 |
| rs2239610 (g.IVS 01 + 63 G>C) | 14.29 | 0.00 | 0.00 | 0.00 | 2 | 12 | 0 | 14 | 0 | 10 | 0 | 32 | 0.18 | 1 | 1 |
| rs2075176 (g.IVS 06–69 C>T) | 35.71 | 7.14 | 10.00 | 6.25 | 5 | 9 | 1 | 13 | 1 | 9 | 2 | 30 | 0.04* | 1 | 1 |
| rs2241119 (g.IVS 06 + 13 A > G) | 35.71 | 7.14 | 10.00 | 6.25 | 5 | 9 | 1 | 13 | 1 | 9 | 2 | 30 | 0.04* | 1 | 1 |
| rs539239352 (g.IVS 09 + 58 T > G) | 7.14 | 0.00 | 0.00 | 0.00 | 1 | 13 | 0 | 14 | 0 | 10 | 0 | 32 | 1 | 1 | 1 |
| rs1991517 (c.2337 C>G) | 42.86 | 28.57 | 0.00 | 9.38 | 6 | 8 | 4 | 10 | 0 | 10 | 3 | 29 | 0.03* | 0.23 | 1 |
| rs2268477 (c.172 C>A) | 28.57 | 14.29 | 50.00 | 0.00 | 4 | 10 | 2 | 12 | 5 | 5 | 0 | 32 | 0.01* | 0.18 | 0.001* |
| rs373305430 (c.182 G > T) | 0.00 | 0.00 | 10.00 | 0.00 | 0 | 14 | 0 | 14 | 1 | 9 | 0 | 32 | 1 | 1 | 0.48 |
| rs7144481 (c.245 C>T) | 78.57 | 57.14 | 100.00 | 0.00 | 11 | 3 | 8 | 6 | 10 | 0 | 0 | 32 | 0.0001* | 0.0001* | 0.0001* |
| rs17630128 (c.431 T>C) | 35.71 | 28.57 | 30.00 | 0.00 | 5 | 9 | 4 | 10 | 3 | 7 | 0 | 32 | 0.003* | 0.01* | 0.02* |
| *TPO* variants | | | | | | | | | | | | | | | |
| rs2280132 (c.1208 G > T) | 7.14 | 0.00 | 10.00 | 9.38 | 1 | 13 | 0 | 14 | 1 | 9 | 3 | 29 | 0.44 | 0.65 | 1 |
| rs2175977 (c.1284 G>C) | 35.71 | 57.14 | 30.00 | 9.38 | 5 | 9 | 8 | 6 | 3 | 7 | 3 | 29 | 0.09 | 0.002* | 0.27 |
| rs140322336 (c.1948 C>G) | 7.14 | 0.00 | 0.00 | 0.00 | 1 | 13 | 0 | 14 | 0 | 10 | 0 | 32 | 1 | 1 | 1 |
| rs1126797 (c.2089 C>T) | 42.86 | 14.29 | 20.00 | 15.63 | 6 | 8 | 2 | 12 | 2 | 8 | 5 | 27 | 0.11 | 1 | 1 |
| rs10189329 (g.IVS11 + 20 G > A) | 0.00 | 0.00 | 0.00 | 6.25 | 0 | 14 | 0 | 14 | 0 | 10 | 2 | 30 | 0.96 | 0.96 | 1 |
| rs867983 (g.IVS13 + 128 C>T) | 71.43 | 42.86 | 50.00 | 21.88 | 10 | 4 | 6 | 8 | 5 | 5 | 7 | 25 | 0.004* | 0.27 | 0.19 |
| rs746074402 (g.IVS14-37 G > A) | 0.00 | 14.29 | 0.00 | 0.00 | 0 | 14 | 2 | 12 | 0 | 10 | 0 | 32 | 1 | 0.18 | 1 |
| Novel (g.IVS14-19 G>C) | 0.00 | 7.14 | 0.00 | 0.00 | 0 | 14 | 1 | 13 | 0 | 10 | 0 | 32 | 1 | 1 | 1 |
| *TG* variants | | | | | | | | | | | | | | | |
| Novel (c.1262 C>T) | 7.14 | 0.00 | 0.00 | 0.00 | 1 | 13 | 0 | 14 | 0 | 10 | 0 | 32 | 1 | 1 | 1 |
| rs2069548 (c.1999 G > A) | 0.00 | 14.29 | 0.00 | 6.25 | 0 | 14 | 2 | 12 | 0 | 10 | 2 | 30 | 0.96 | 0.71 | 1 |
| rs2069550 (c.2375T>C) | 28.57 | 14.29 | 0.00 | 6.25 | 4 | 10 | 2 | 12 | 0 | 10 | 2 | 30 | 0.1 | 0.71 | 1 |
| rs2076740 (c.6036 C>T) | 64.29 | 35.71 | 30.00 | 3.13 | 9 | 5 | 5 | 9 | 3 | 7 | 1 | 31 | 0.0001* | 0.014* | 0.07 |

*MAF* minor allele frequencies, *M* mutant allele, *W* wild allele

*Statistically significant

**Table 2** Association of *TSHR* SNPs with risk for CH in toto

| rs number | Nucleotide change | CH Cases | | | | Controls | | | | OR | 95% CI | p value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | WW | WM | MM | MAF | WW | WM | MM | MAF | | | |
| rs2234919 | c.310 C>A | 40 | 5 | 0 | 5.56 | 483 | 190 | 27 | 17.43 | 0.28 | 0.11–0.69 | 0.002* |
| rs2239610 | g.IVS 01 + 63 G>C | 44 | 0 | 1 | 2.22 | 482 | 174 | 44 | 18.71 | 0.1 | 0.02–0.40 | < 0.0001* |
| rs2075176 | g.IVS 06–69 C>T | 38 | 5 | 2 | 10.00 | 477 | 196 | 27 | 17.86 | 0.51 | 0.25–1.03 | 0.07 |
| rs2241119 | g.IVS 06 + 13 A > G | 38 | 5 | 2 | 10.00 | 486 | 188 | 26 | 17.14 | 0.54 | 0.27–1.08 | 0.09 |
| rs539239352 | g.IVS 09 + 58 T > G | 44 | 1 | 0 | 1.11 | 691 | 9 | 0 | 0.64 | 1.74 | 0.22–13.86 | 0.93 |
| rs1991517 | c.2337 C>G | 31 | 9 | 5 | 21.11 | 595 | 92 | 13 | 8.43 | **2.91** | **1.69–4.99** | < 0.0001* |
| rs2268477 | c.172 C>A | 35 | 8 | 2 | 13.33 | 644 | 24 | 32 | 6.29 | **2.29** | **1.20–4.37** | 0.03* |
| rs373305430 | c. 182 G > T | 44 | 1 | 0 | 1.11 | 692 | 2 | 6 | 1.00 | 1.11 | 0.15–8.56 | 1 |
| rs7144481 | c.245 C>T | 29 | 1 | 15 | 34.44 | 557 | 13 | 130 | 19.50 | **2.17** | **1.38–3.42** | 0.002* |
| rs17630128 | c.431 T>C | 37 | 5 | 3 | 12.22 | 675 | 9 | 16 | 2.93 | **4.62** | **2.29–9.32** | < 0.0001* |

The bold values signify positive association with the congenital hypothyroidism

*WW* wild, *WM* heterozygous, *MM* homozygous, *MAF* minor allele frequency, *OR* odds ratios, *CI* confidence intervals

*Statistically significant

p = 0.0001) and goiter (MAF: 35.71% vs. 3.13%, p = 0.01) (Table 1).

### *DUOX2* variants

Exon 12 and 16 of the *DUOX2* gene along with splicing regions were analyzed by sequencing. The sequencing results revealed that all cases had only the wild type sequence in the *DUOX2* gene.

## Discussion

The current study established Indian population-specific reference ranges for TSH in male and female neonates. Further, a slight elevation in TSH was noticed in neonates with lower or higher birth weight compared to those with optimal birth weight. Thyroid scan of 45 confirmed CH cases revealed agenesis in seven, hypoplasia in five and goiter in seven and no specific abnormality of the thyroid gland in 16 while 10 cases didn't opt for thyroid scan. All the cases were evaluated for 22 genetic variants in three candidate genes, i.e. *TSHR*, *TPO* and *TG*. Heat map analysis and Fisher exact tests were conducted to establish genotype–phenotype associations. Among the *TSHR* variants, rs7144481 and rs17630128 showed association with agenesis, hypoplasia and goiter. The rs2268477 variant showed association with hypoplasia and agenesis, while rs1991517 showed association only with agenesis. The four *TSHR* variants, i.e. rs714481, rs17630128, rs2268477, and rs1991517 showed association with CH in toto. The rs2075176 and rs2241119 variants showed borderline association with agenesis. Among the eight *TPO* variants, only rs2175977 and rs867983 showed association with goiter and agenesis, respectively. Among the four *TG* variants, only rs2076740 showed association with agenesis and goiter. No genetic variants found in exon 12 and 16 of *DUOX2*. In silico studies revealed the deleterious nature of two variants.

The results of the current study corroborate with Grob et al., in demonstrating higher TSH levels in preterm newborns small for gestation age [19]. A targeted next generation sequencing based study of 43 Chinese CH cases demonstrated *TSHR*, *TPO*, *TG* and *DUOX2* as the most likely candidate genes affected in CH out of 29 causative genes consistent with our objective of the study [20]. The rs7144481 variant was one of the three variants identified in a genome wide association study as a genetic contributor to elite endurance performance [21]. Null association of rs7144481, rs17630128 and rs2268477 variants with papillary thyroid cancer was reported earlier [22]. The current study demonstrated the association of rs1991517 with agenesis, which in turn has lower TSH levels compared to other CH thyroid phenotypes. This phenomenon was reported earlier, even in healthy subjects with heterozygous exhibiting lower TSH values than those with wild genotype [23].

Among the *TPO* variants, rs2175977 was associated with goiter. This variant was reported earlier in five cases of CH cases in Baghdad [24]. There are no studies to corroborate our findings with rs867943. The *TG* rs2076740 polymorphism was earlier reported to be a significant genetic risk factor for autoimmune thyroid disease based on extensive meta-analysis of 3013 cases and 1812 controls [25].

In addition to the known variants, we have identified two rare genetic variants, i.e. *TPO* g.IVS14-19 G>C and *TG* c.1262 C>T.

The strengths of the current study are: (i) establishment of reference ranges for TSH in neonates by considering gender and birth weight; (ii) exploration of 22 SNVs in three

candidate genes to establish genotype–phenotype association in CH cases; (iii) the association of *TSHR* variants with CH in toto was established by analyzing 700 screen negative newborns. The limitations are: (i) all the exons and introns were not analyzed in these candidate genes and hence likely to miss rare variants; (ii) the distribution of other variants in screening negative newborns was not evaluated.

To conclude, the current study established reference ranges for TSH in neonates by segregating according to gender and birth weight. Mild elevation of TSH was observed in neonates with low or high birth weight compared to those with optimal weight. The confirmed CH cases exhibited specific genotype–phenotype associations with *TSHR*, *TPO* and *TG* SNVs. *DUOX2* genetic variants showed a null association.

**Acknowledgements** Authors specially thank Dr. Pasumarthi NBS Srinivas, Anusha Puvvada, Uma Maheshwar P for their support during the investigation.

**Author contributions** VRD, RRDA and SMN designed the work. The work was primarily executed by YK under the supervision of VRD, RRDA and BRG. SMN, DB and MT performed the analysis and interpreted the data. SMN, SN, LL, RKP and BRG gave constructive comments during execution of the work. The manuscript was written by YK and SMN with extensive support of VRD and RRDA. All the authors have given important insights and approved the final version of manuscript.

**Funding** This work was partly supported by a grant from DST-SERB, Government of India (ECR/2016/00304).

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** The study was performed in accordance with the ethical standards of the Institutional Ethics committee of Rainbow Hospital Institutional Ethics Committee (RCHBH/066/02-2018), Hyderabad, India and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

**Informed consent** Informed written consents were obtained from parents and participants of all subjects included in the study.

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