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Genetic testing can change diagnosis and treatment in children with congenital hypothyroidism

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Abstract

Objective: Guidelines on congenital hypothyroidism (CH) recommend that genetic testing should aim to improve diagnosis, treatment or prognosis, but it is unclear which patients would benefit most from the genetic investigation. We aimed to investigate the genetic etiology of transient CH (TCH) and permanent CH (PCH) in a well-characterized cohort, and thereby evaluate the impact of genetic testing on the management and prognosis of children with CH.

Methods: A total of 48 CH patients with normal, goitrous ($n = 5$) or hypoplastic thyroid ($n = 5$) were studied by high-throughput sequencing using a custom-designed 23-gene panel. Patients initially categorized as TCH ($n = 15$), PCH ($n = 26$) and persistent hyperthyrotropinemia (PHT, $n = 7$) were re-evaluated after genetic testing.

Results: Re-evaluation based on genetic testing changed the initial diagnoses from PCH to PHT ($n = 2$) or TCH ($n = 3$) and from PHT to TCH ($n = 5$), which resulted in a final distribution of TCH ($n = 23$), PCH ($n = 21$) and PHT ($n = 4$). Genetic analysis also allowed us to discontinue treatment in five patients with monoallelic *TSHR* or *DUOX2*, or no pathogenic variants. The main reasons for changes in diagnosis and treatment were the detection of monoallelic *TSHR* variants and the misdiagnosis of thyroid hypoplasia on neonatal ultrasound in low birthweight infants. A total of 41 (35 different, 15 novel) variants were detected in 65% ($n = 31$) of the cohort. These variants, which most frequently affected *TG*, *TSHR* and *DUOX2*, explained the genetic etiology in 46% ($n = 22$) of the patients. The molecular diagnosis rate was significantly higher in patients with PCH (57%, $n = 12$) than TCH (26%, $n = 6$).

Conclusions: Genetic testing can change diagnosis and treatment decisions in a small proportion of children with CH, but the resulting benefit may outweigh the burden of lifelong follow-up and treatment.

Key Words

- ▶ congenital hypothyroidism
- ▶ genetic testing
- ▶ persistent hyperthyrotropinemia
- ▶ thyroid hypoplasia
- ▶ TSH resistance

Introduction

Primary congenital hypothyroidism (CH) is the most common endocrine disorder in newborns, with an incidence ranging from 1/1000 to 1/3000 worldwide (1). The incidence of CH has doubled over the last two decades largely due to the more frequent detection of mild and transient CH (TCH) cases with a gland *in situ* (GIS) (2, 3). With increasing incidence, the etiologic spectrum of CH has also changed, and the proportion of patients with GIS has increased to 35–40% (3, 4). Primary CH with GIS (GIS-CH) is caused by dysmorphogenesis, defects of hormone synthesis within normally located, sometimes goitrous thyroid gland. It is usually due to autosomal recessive mutations in *TG*, *TPO*, *DUOX2*, *DUOXA2*, *SLC26A4* (*Pendrin*), *SLC5A5* (*NIS*), *IYD* and *SLC26A7*. Also, monoallelic variants in *DUOX2* and *DUOXA2* can cause TCH. Thyroid dysgenesis (TD), now responsible for 60–65% of primary CH, refers to a phenotypic spectrum of aberrant thyroid gland development and includes athyreosis, ectopy, orthotopic hypoplasia and hemigenesis. TD can result from mutations that are inherited in an autosomal dominant (*PAX8*, *NKX2-1*, *NKX2-2*, *JAG1*, *NTN1*), recessive (*FOXE1*, *GLIS3*) or both (*CDCA8*, *TSHR*) manner (4). Resistance to thyrotropin (TSH) caused by monoallelic or biallelic *TSHR* variants has a special place in the etiology of primary CH, with a phenotype varying from mild persistent hyperthyrotropinemia (PHT) with normal-sized gland to severe permanent CH (PCH) with orthotopic hypoplasia (4, 5, 6). Depending on the deleteriousness and number of mutated *TSHR* alleles, TSH resistance may cause GIS-CH or TD.

The studies on the genetics of CH have increased with the advent of high-throughput sequencing (HTS), which allows simultaneous and systematic analysis of known and candidate genes associated with various diseases. The recent HTS studies have been useful in elucidating the genetic causes of CH, with the findings that most cases of CH with GIS and thyroid hypoplasia have an identifiable genetic basis, but only less than 5% of the patients with ectopy and athyreosis harbor causative mutations (7, 8, 9, 10, 11, 12). However, none of these studies focused on the contribution of genetic testing to patient management, nor did they address patients with TCH. Current consensus guidelines on CH recommend that genetic testing should aim to improve diagnosis, treatment or prognosis of the patients (13). But it is unclear which patients would benefit most from such a genetic investigation. Therefore, we aimed to investigate

the genetic etiology of TCH, PCH and PHT in a well-characterized cohort and thereby evaluate the impact of genetic testing on the management and prognosis of patients with CH.

Materials and methods

Patients and diagnostic criteria for PCH, TCH and PHT

The Ethics Committee of Ondokuz Mayıs University approved the study (Protocol No 57 dated 09.02.2017). Informed written consent was obtained from the parents of each patient. Primary CH patients with normal, goitrous or hypoplastic thyroid were included in the study, but those with TD due to ectopy and athyreosis were ruled out. This selection aimed to use financial resources more efficiently by studying CH patients with a more identifiable genetic basis. Patients not yet categorized as TCH or PCH, diagnosed with TCH before 2 years of age and with insufficient dataset were excluded. In familial CH cases, only the proband was enrolled. Among the patients ($n = 106$) who were eligible for the study, 67 applied to our clinic for follow-up examination during the recruitment period between 1 April and 30 September 2017. Only 48 out of 67 patients gave parental consent for participation and thereby were recruited in the genetic study (Fig. 1). Demographic and clinical characteristics of patients not included in the genetic study were similar to those included (data not presented).

Diagnosis of primary CH was based on the national newborn screening program implemented in December 2006. The blood spot TSH cut-off value in the program was initially 10 $\mu\text{U/mL}$ (serum equivalent 20 $\mu\text{U/mL}$). It was lowered to 7.5 $\mu\text{U/mL}$ in 2009, and from 2012 until present, the cut-off is 5.5 $\mu\text{U/mL}$ (14, 15). Diagnosis of CH was confirmed by serum TSH and free thyroxine (FT4) measurements, according to laboratory reference values (see supplementary material for reference ranges). All CH patients with low serum FT4 and/or persistently high (>10 $\mu\text{U/mL}$) TSH were treated with L-thyroxine (LT4). Two patients born before neonatal screening were diagnosed due to clinical symptoms suggesting hypothyroidism. At diagnosis, the position and size of the thyroid gland were evaluated by scintigraphy ($n = 14$), ultrasound (US, $n = 27$) or both ($n = 7$). Also, all the patients re-evaluated through LT4 therapy withdrawal were undergone routine thyroid US examination ($n = 34$).

The patients were initially divided into three diagnostic groups as PCH, TCH and PHT at presentation,

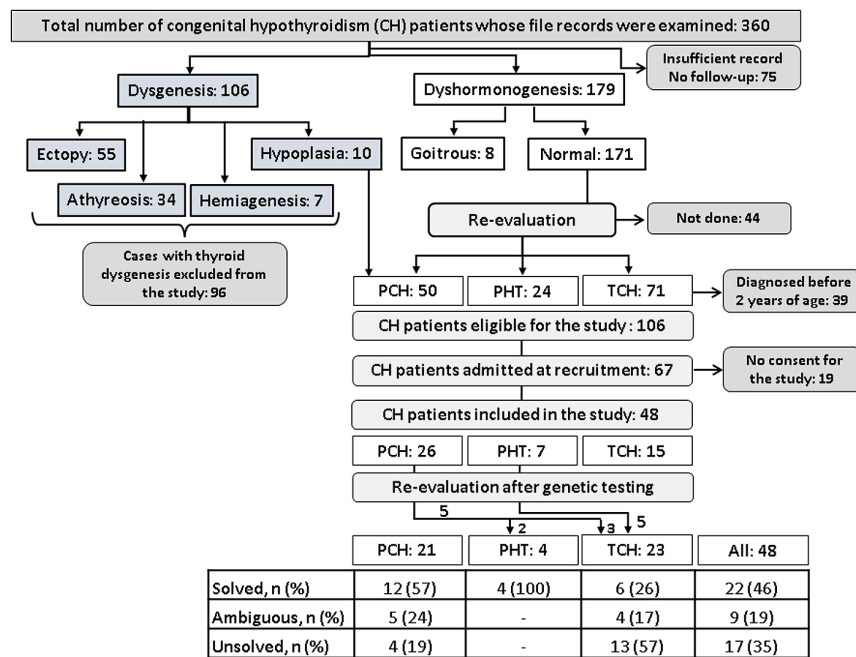


Figure 1

Schematic illustration of case selection, diagnoses before and after genetic testing and the proportions of solved, ambiguous and unsolved cases. Primary CH patients with normal, goitrous or hypoplastic thyroid were included in the study, but patients with ectopy, athyreosis and hemiagenesis were ruled out. Patients with hypoplastic thyroid and those with FT4 decline and/or TSH rise (>10 µU/mL) on treatment or after trial off LT4 constituted the PCH group. Patients whose TSH levels were within normal limits (<5 µU/mL) or mildly elevated (5–10 µU/mL) despite normal FT4 levels at re-evaluation were classified as TCH or PHT, respectively. Patients not yet categorized as TCH or PCH, diagnosed with TCH before 2 years of age and with insufficient dataset were excluded. Out of 106 eligible patients, 67 applied for the follow-up visit during recruitment, and only 48 gave consent for participation in the genetic study. After clinical re-evaluation based on genetic test results, the definitive diagnoses were changed from PCH to PHT (n: 2) or TCH (n: 3) and from PHT to TCH (n: 5) in 10 patients, and LT4 was discontinued in 5 patients. Finally, the genotype–phenotype correlations were analyzed. The molecular diagnosis rate (proportion of ‘solved’ cases) was 46% in the whole cohort. But, it was significantly higher in patients with PCH (57%) than TCH (26%). CH, congenital hypothyroidism; LT4, L-thyroxine; PCH, permanent CH; PHT, persistent hyperthyrotropinemia; TCH, transient CH.

during follow-up or at re-evaluation after the age of 2–3 years. The patients were included in the PCH group if they had any of the following criteria: (i) TD on scan or US at presentation, (ii) an increase in LT4 dosage over time due to TSH rise on treatment or (iii) low FT4 and/or elevated TSH levels after treatment withdrawal (TSH > 15 µU/mL at 1st month or >10 µU/mL at or after 3rd month) at re-evaluation after 2–3 years of age (16, 17). In patients whose TSH levels were suppressed despite minimally reduced doses of LT4 (≤12.5 µg/day), the therapy was withdrawn from 6 months of age to avoid overtreatment (13). The patients with normal FT4 and TSH levels (<5 µU/mL) after treatment withdrawal were classified as TCH, while those with mildly elevated TSH levels (5–10 µU/mL) despite normal FT4 were diagnosed with PHT (17). TCH cases requiring at least 2 years of LT4 treatment were included in the genetic study, while the others diagnosed before 2 years of age were excluded, considering that environmental factors would be dominant in the etiology of this group (18).

After genetic analysis, we reassessed all patients for genotype–phenotype correlation and made a trial off treatment in selected patients who were initially classified

as PCH or PHT, but had monoallelic variants in *DUOX2*, *DUOX2A2*, *TG* and/or *TSHR* genes, or no pathogenic variant. This selection was based on the following data: (i) the causality of *DUOX2* and *DUOX2A2* variants with TCH (18), (ii) autosomal recessive inheritance of *TG* variants in general (19) and (iii) the reports on the association of monoallelic *TSHR* variants with well-compensated PHT, regardless LT4 treatment (5, 6). Therefore, the definitive diagnoses were established according to the ultimate clinical assessment after genetic testing (Fig. 1).

Finally, we analyzed the genotype–phenotype correlations. If there is a decisive link between genotype and phenotype, these patients were considered to be ‘solved’. If genetic variants contribute reasonably to the phenotype but of which the evidence of a causal link was weaker than in the solved group, they were categorized as ‘ambiguous’ (7). Patients with no pathogenic variant in any of the targeted genes were grouped as ‘unsolved’ (Fig. 1, Supplementary Table 2, see section on supplementary materials given at the end of this article).

Anthropometric measurements, thyroid volumes and standard deviation (s.d.) scores were calculated with an online calculation program using reference data from

Turkish children (<https://www.childmetrics.org/>) (20). Thyroid volume s.d. scores were assessed according to age and gender-specific references (21, 22) Data were expressed as mean (\pm s.d.), median (interquartile range) or percentage (%), as appropriate.

DNA sequencing

Genomic DNA was extracted from peripheral leukocytes using QIAamp DNA Blood Mini Kits (Qiagen), and stored at -20°C up to the time of analysis. A custom HTS panel (GeneSGKit[®], Sistemas Genomicos, Valencia, Spain) targeting 23 genes known to cause CH was used for DNA library preparation (Supplementary Table 1). Libraries including all coding regions and 10 bp exon-intron boundaries of the targeted genes were sequenced on the MiSeq instrument (Illumina, San Diego, CA, USA) according to the manufacturer's protocol. Bioinformatics analysis was carried out using GeneSystem variant analysis platform (Sistemas Genomicos). All filtered positive results of the HTS were confirmed by Sanger sequencing using 3500xL Genetic Analyzer (Applied Biosystems). Parental DNA samples were included with Sanger sequencing for segregation analysis, particularly in patients for whom the genetic etiology of CH remained unclear.

The 23-gene HTS panel covered, with a minimum read depth of 20 \times , 100% of the targeted gene regions of all patients, except for two (#4 and #8) who had relatively low DNA concentrations. The mean read depth in all patients was 270. In #4 and #8, the coverage percentages were 99.994 and 99.993%, and the read depths were 55 and 41, respectively (Supplementary Table 1). Therefore, we performed PCR amplifications and Sanger sequencing of the uncovered regions in these two patients.

Variant filtering, classification and in silico analysis

Nonsense, frameshift, splice site and missense variants with a minor allele frequency < 1% were taken into consideration for variant analysis. The frequency of the identified variants was checked in population databases including NCBI dbSNP, 1000 Genomes Project, Exome Aggregation Consortium and Genome Aggregation Database. Variants were described in accordance with the nomenclature recommended by the Human Genome Variation Society (<https://varnomen.hgvs.org>) (23). The functional annotations of the identified variants were validated using Varsome database (<https://www.varsome.com>) and classified according to standards defined by

the 'American College of Medical Genetics' (24). Likely benign or benign variants were excluded from the analysis. The identified variants were also checked using the 'Human Gene Mutation Database' (HGMD) whether they were reported before (25). Variants recorded in the HGMD Professional were classified as 'known' and the others as 'novel'. Finally, the pathogenicity of variants was analyzed using 30 different *in silico* prediction algorithms including SIFT, Polyphen2 and MutationTaster2 (see the Supplementary Materials for whole list). Variants annotated as deleterious in most of the prediction algorithms were classified as potentially pathogenic, which means supporting pathogenicity (24).

Results

The study group consisted of 48 unrelated patients, 31 males and 17 females. The median age at diagnosis was 17 (11–26) days. Consanguinity was found in 40% of the cohort. Familial history of CH was present in 10 patients (21%). The rates of preterm birth and born small for gestational age (SGA) were 14.6 and 10.4%, respectively. All demographic, clinical and laboratory characteristics of the reclassified cohort are comparatively presented in Table 1.

Based on initial clinical data, the patients were classified as PCH ($n=26$), PHT ($n=7$) and TCH ($n=15$). Re-evaluation after genetic testing led to changes in diagnostic categories in 10 patients, with a final distribution of PCH ($n=21$), PHT ($n=4$) and TCH ($n=23$) (Fig. 1, Table 2). Genetic test results such as monoallelic *DUOX2*, *TG* and/or *TSHR* variants, or absence of pathogenic variant prompted us a trial off medication for the first time in seven patients initially classified as PCH. The reasons for not previously discontinuation of LT4 were as follows: a finding of hypoplastic thyroid on neonatal US (#19, #24), elevated TSH values at times of nonadherence (#16, #30, and #32) or during even regular treatment (#7, #46). Among patients reassessed after genetic testing, two with monoallelic *TG* variants (#30, #32) required therapy, and so the diagnosis of PCH remained unchanged. However, the other five patients did not require therapy. Of these, two (#7, #46) with heterozygous *TSHR* variants had well-compensated hyperthyrotropinemia and were reclassified as PHT. Also, three patients (#19 with monoallelic *DUOX2* variant, and #16 and #24 with no pathogenic variant) were reclassified as TCH (Table 2, Supplementary Table 2).

Of seven patients initially classified as PHT, two (#11, #39) were explained by monoallelic *TSHR* mutations.

Table 1 Demographic, clinical and laboratory characteristics of the reclassified cohort.^a

Parameter	PCH-PHT ^b (n = 25)	TCH (n = 23)	P value
Sex: male/female (n)	16/9	15/8	0.93
Age at diagnosis (day)	19 (12–35)	16 (11–21)	0.14
Gestational age (week)	40 (39–40)	38 (37–40)	0.02
Birth weight (g)	3259 ± 553	2982 ± 807	0.17
Prematurity (%)	8	22	0.24
SGA birth (%)	4	17	0.18
Consanguinity (%)	48	30	0.25
Family history of CH (%)	36	4	0.01
Associated feature (n)	Renal cyst (1) Hypospadias (1) Esotropia (1)	ASD-VSD (1) Hearing loss (1) Strabismus (1)	-
TSH (RR: 0.72–11.0 µU/mL) ^c	100 (53.7–100)	100 (40.4–100)	0.12
Free T4 (RR: 0.89–2.2 ng/dL) ^c	0.62 ± 0.33	0.81 ± 0.39	0.04
Free T3 (RR: 1.95–6.0 pg/mL) ^c	2.97 ± 1.29	4.02 ± 2.06	0.12
Thyroglobulin (RR: 20–228 ng/mL)	5.0 (1.3–17.8)	300 (142–300)	<0.001
Thyroid volume s.d. score on US	−0.37 (−1.69 to 0.93)	−0.55 (−0.96 to 0.42)	0.72
Thyroid morphology on US (n = 34) and/or scan (n = 21) Goiter/normal/hypoplasia (n)	5/15/5	0/23/0	<0.001
LT4 dose at diagnosis (µg/kg/day)	9.6 ± 4.2	10.2 ± 3.2	0.58
LT4 dose at last visit or withdrawal time (µg/kg/day)	3.4 ± 1.1	1.4 ± 0.4	<0.001
Trial off LT4 (n)	11	23	<0.001
Age at LT4 withdrawal (years)	6.2 (3.5–8.6)	3.1 (2.7–3.8)	0.02
TSH at 1st month of trial off (RR: 0.70–5.97 µU/mL) ^c	11.6 (9.0–17.9)	4.3 (2.9–7.8)	<0.001
TSH at 1st year of trial off (RR: 0.70–5.97 µU/mL) ^c	11.1 (7.8–17.8) ^d	4.2 (2.8–4.7)	0.001

^aData were expressed as mean (±s.d.), median (interquartile range) or percentage (%). Two groups were compared using parametric (chi-square and Student's t) or nonparametric (Fisher's exact and Mann-Whitney U) tests, as appropriate. P values less than 0.05 were considered statistically significant; ^bFor statistical evaluations, the patients with PCH (n: 21) and PHT (n: 4) were combined into one group; ^cReference ranges are given for infants between 6 days and 3 months and for children between 1 and 7 years, covering the ages at diagnosis and LT4 withdrawal, respectively. To convert free T4 and free T3 values to Système Internationale (SI) units, multiply by 12.87 and 1.54 (to pmol/L), respectively; ^dUntreated PHT patients with monoallelic *TSHR* variants (n: 4). CH, congenital hypothyroidism; LT4, levothyroxine; RR, reference range; Scan, scintigraphy; SGA, small for gestational age; T3, tri-iodothyronine; T4, thyroxine; TSH, thyroid-stimulating hormone; US, ultrasound.

Table 2 Impact of genetic testing on definitive diagnoses and treatment.

Case No	Gene	Variant ^a	Diagnosis before HTS	Diagnosis after HTS	Age at trial off therapy	Age at last visit	Treatment
#7	<i>TSHR</i>	D474E	PCH	PHT	8.5	10.5	Discontinued
#46	<i>TSHR</i>	D474E	PCH	PHT	9.1	11.0	Discontinued
#16	-	-	PCH	TCH	4.4	6.8	Discontinued
#19	<i>DUOX2</i>	R1470W	PCH	TCH	8.4	9.9	Discontinued
#24	-	-	PCH	TCH	6.5	8.0	Discontinued
#30	<i>DUOX2</i>	R434*	PCH	PCH	8.5	9.3	Resumed
	<i>TG</i>	c.638+5G>A					
#32	<i>TG</i>	W1050L	PCH	PCH	10.3	11.5	Resumed
#11	<i>TSHR</i>	P68S	PHT	PHT	2.0	4.5	Not restarted
	<i>DUOXA2</i>	c.769+2T>A					
#39	<i>TSHR</i>	D474E	PHT	PHT	4.0	6.0	Not restarted
#10	-	-	PHT	TCH	3.8	9.1	Not needed
#14	-	-	PHT	TCH	2.8	6.8	Not needed
#17	<i>GNAS</i>	D464N	PHT	TCH	3.2	5.9	Not needed
#38	-	-	PHT	TCH	2.6	5.0	Not needed
#45	<i>GNAS</i>	A314T	PHT	TCH	3.3	7.7	Not needed

^aAll variants were heterozygous.

CH, congenital hypothyroidism; HTS, high-throughput sequencing; PCH, permanent CH; PHT, persistent hyperthyrotropinemia; TCH, transient CH.

However, the other five (#10, #14, #17, #38 and #45) had no causative variant. We checked these patients for thyroid function at ages 5–9 years and observed that their TSH levels returned to normal 2.5–5 years after LT4 cessation. Therefore, these five patients were reclassified as TCH (Fig. 1, Table 2).

The identified variants have definitely explained the genetic etiology in 46% ($n=22$) of the cohort, so this group were considered as solved. Genetic etiology was accepted as ambiguous in 19% patients ($n=9$). The others with no identified pathogenic variant remained unsolved. The molecular diagnosis rate was significantly higher in patients with PCH (57%) than TCH (26%). All four patients with PHT were explained by heterozygous *TSHR* variants (Fig. 1).

A total of 41 variants (31 monoallelic, 10 biallelic) were detected in 65% ($n=31$) of all the patients (Table 3). Of these patients, eight (26%) had oligogenic variants. A missense variant D474E (c.1422C>A) in *TSHR* gene was detected as biallelic ($n=2$) or monoallelic ($n=3$) in five patients with PCH, making it the most frequently identified genetic variant in our cohort. Also, two couples of patients with PCH harbored the same splice site variants in the *TG* gene. Thus, we identified 35 different (15 novel) variants (Supplementary Table 2).

The genes most frequently affected by the variants were *TG* (27%), *TSHR* (20%) and *DUOX2* (15%). The other genes carrying variants were *TPO*, *DUOX2*, *PAX8*, *FOXE1*, *SLC26A4*, *SLC5A5*, *IYD* and *GNAS* (Table 3). However, no

pathogenic variant was detected in any of the other TD and central or peripheral hypothyroidism genes included in the HTS panel (Supplementary Table 1). The most commonly mutated genes in PCH were *TSHR* and *TG*, followed by *TPO* and *PAX8*. Monoallelic *DUOX2-DUOX2* variants underlay TCH (Table 3, Supplementary Table 2).

Discussion

The genetic etiology of GIS-CH was identified in 46% of the cohort using a custom-designed 23-gene HTS panel. Molecular diagnosis rate was significantly higher in the patients with PCH (57%) than TCH (26%). A diagnostic yield of 57% in PCH patients is consistent with the recent HTS data ranging from 40 to 65% (7, 8, 9, 10, 11, 12). Unlike previous studies, we used genetic data not only for research but also in daily practice, thus having the chance to observe how molecular diagnosis can change clinical decisions on diagnosis and treatment and may improve the management and prognosis of the patients with GIS-CH.

Genetic testing led to changes in definitive diagnoses and allowed us to cease LT4 therapy in five patients, which represent 10% of the cohort. These data reflect the impact of genetic testing on clinical outcomes and prognosis but raise some questions. What are the reasons for changes in clinical diagnoses or treatment decisions? Was it possible to avoid these mistakes without genetic

Table 3 Variant frequency in targeted genes by diagnostic groups of PCH-PHT and TCH.

Gene group ^a	Gene	PCH-PHT ^b ($n=25$)		TCH ($n=23$)		Variant frequency n (%)
		Monogenic	Oligogenic	Monogenic	Oligogenic	
Dyshormonogenesis	<i>TG</i>	4 Hom, 5 Het	1 Het	0	1 Het	11 (27)
	<i>DUOX2</i>	0	2 Het	4 Het	0	6 (15)
	<i>TPO</i>	1 Hom, 1 Comp	0	0	0	3 (7)
	<i>DUOX2</i>	0	1 Het	1 Het	0	2 (5)
	<i>SLC26A4</i>	0	1 Het	1 Hom	0	2 (5)
	<i>SLC5A5</i>	0	0	0	1 Het	1 (2)
	<i>IYD</i>	0	1 Het	0	0	1 (2)
	<i>DUOX1</i>	0	0	0	0	0
Resistance to thyrotropin	<i>TSHR</i>	4 Hom, 4 Het	0	0	0	8 (20)
	<i>GNAS</i>	0	0	2 Het	1 Het	3 (7)
Thyroid dysgenesis	<i>PAX8</i>	2 Het	0	0	0	2 (5)
	<i>FOXE1</i>	0	1 Het	1 Het	0	2 (5)
	<i>NKX2-1</i>	0	0	0	0	0
	<i>NKX2-5</i>	0	0	0	0	0
	<i>GLIS3</i>	0	0	0	0	0
Total number of variants		29		12		41 (100)

^aThe table does not include central and peripheral CH genes, in which no pathogenic variant was detected; ^bThe patients with PCH ($n=21$) and PHT ($n=4$) were combined into one group.

CH, congenital hypothyroidism; Comp, compound heterozygous; Het, heterozygous; Hom, homozygous; PCH, permanent CH; PHT, persistent hyperthyrotropinemia; TCH, transient CH.

testing? Upon review of the data, we attributed the changes in diagnosis and treatment to three different reasons. Foremost, genetic data directly guided patient management in two cases (#7, #46). Elevated TSH values under treatment did not allow the re-evaluation of these patients. After obtaining data that they had monoallelic *TSHR* variants (D474E), we tried to withdraw treatment and found that serum FT4 levels remained within normal limits despite moderately high TSH levels during follow-up. Tenenbaum-Rakover *et al.* studied the long-term outcome of loss-of-function mutations in *TSHR* gene and showed that subclinical hypothyroidism in heterozygous subjects is a stable compensated condition with an appropriately adjusted pituitary TSH set point and does not require replacement therapy (5). In our cohort, monoallelic *TSHR* variants (P68S and D474E) were detected in other two children (#11, #39) whose treatment was previously discontinued due to mild PHT. All heterozygotes with *TSHR* variants were clinically asymptomatic and had normal growth during long-term follow-up. So, we did not resume LT4 therapy in these children.

The second reason for diagnostic changes was the normalization of TSH levels in PHT cases with no causative variant. In five cases, TSH values returned to normal between the ages of 5 and 9. In fact, even without genetic data, this outcome could have been obtained with a sufficient follow-up period. Leonardi *et al.* monitored thyroid function tests of 44 children with mild PHT detected at neonatal screening and found that TSH levels were normalized in most (68%) of children until 9 years old (26). In that study, the children with ongoing PHT or ‘subclinical hypothyroidism’ at prepubertal age had a thyroid morphological and/or genetic abnormality. As a result, our data support that TSH levels may return to normal until late childhood in PHT patients whose thyroid imaging and genetic testing were normal.

Another reason was the incorrect evaluation of thyroid morphology on neonatal US. Due to a diagnosis of thyroid hypoplasia, two patients (#19, #24) were not subjected to a trial off LT4. After obtaining genetic test results displaying a monoallelic *DUOX2* variant and no pathogenic variant, we reinvestigated thyroid size and found thyroid volumes to be within normal limits. After discontinuation of LT4, the patients remained euthyroid throughout 1.5-year follow-up. If genetic analysis data were not available, these two patients would have been treated for lifelong due to ‘apparent’ thyroid hypoplasia. Reassessment is unnecessary in CH patients with ectopic thyroid or athyreosis; however, the guidelines do not

offer a clear recommendation for patients with thyroid hypoplasia (13, 27). Rabbiosi *et al.* have shown that a finding of thyroid hypoplasia on US was correlated with PCH, and no patients with hypoplastic thyroid harbored TCH (17). Karakoc-Aydiner *et al.* have found that the sensitivity and specificity for US to detect thyroid hypoplasia at the time of initial diagnosis were 100 and 80.4%, respectively (28). The main difficulty in diagnosing hypoplasia by US is the paucity of normative data for thyroid volumes in newborn infants (28). Returned to our patients, we noticed that one was borderline preterm and the other was born SGA. As our reference data did not include preterm or low birthweight infants (21, 22), we realized that thyroid volume s.d. scores might have been miscalculated. As a result, this very instructive experience suggests that thyroid US should be repeated after infancy in order to exclude possible previous measurement or evaluation errors and that the decision on the permanence of CH should not be based solely on the finding of hypoplastic thyroid on neonatal US.

Contrary to the examples above, a trial off treatment based on genetic data did not change the outcome in two patients. Patient #30 harbored a monoallelic variant in each genes *TG* and *DUOX2*, and patient #32 had a monoallelic *TG* variant (W1050L). Patient #34 also carried two monoallelic variants in the gene *TG* and *SLC26A4*. Together with the other three (#23, #29, #41), a total of five patients with PCH had variants of at least two genes, reflecting oligogenic involvement in 24% of PCH patients. This figure is very consistent with the data of the study by de Filippis *et al.* that reveal oligogenic origin in 22% of PCH patients (8). Indeed, all recent HTS studies suggest that oligogenic effects may play an important role in the pathogenesis of CH (7, 8, 9, 10, 11).

Interestingly, *TG* was the gene most frequently affected by variants in our PCH group. In addition to two patients with oligogenic inheritance discussed earlier, we identified monogenic heterozygous *TG* variants in three patients (#25, #32, #44). In one of them (#32), a family screening for p.W1050L variant showed that the pathogenic variation was co-segregated with the phenotype, suggesting it is likely causative for CH. Thyroglobulin deficiency is generally inherited in an autosomal recessive manner, and affected patients have either homozygous or compound heterozygous variants (19), as seen in our four patients. So thyroid dysfunction is not expected in heterozygous individuals. However, patients with heterozygous *TG* variants have been reported in many studies on goiter and CH (9, 10, 29, 30). In fact, molecular studies suggest that different *TG* mutations

may predispose heterozygotes to mild hypothyroidism and goiter (31, 32). Therefore, we thought that heterozygous *TG* variant W1050L might be responsible for a mild phenotype in patient #32 with familial PCH. For confirmation of this hypothesis, further genetic and functional studies are needed to reveal the possible effects of currently identified variants and other non-coding region or copy number variants. Other candidate genes not included in our panel should be also studied, such as the *SLC26A7* gene, which was recently identified as a novel cause of thyroid dysmorphogenesis (33).

In the TCH group, genetic etiology was found at a lower rate. The etiology of TCH may be genetic, environmental or a combination of both, but their relative contribution is unclear and likely differ between geographical cohorts (18). Our previous study on the natural course of CH detected at newborn screening revealed a TCH prevalence of 52% (3). Therefore, we allocated near half of the study group to the transient cases. Despite the small case number, the current study provides for the first time epidemiologic data on the genetic basis of TCH in Turkey. In fact, there is scarce data on the relative contribution of genetic factors to the pathogenesis of TCH. For instance, the studies from Korea (34) and Japan (35) showed that 33–50% of the patients with TCH had mono- and biallelic *DUOX2* variants. Already, *DUOX2* variants are the most common cause of PCH in Far Eastern countries (9, 10, 34, 35). Interestingly, in a British cohort of predominantly Asian descent, pathogenic mutations in *DUOX2/DUOX2A2* genes were found to be as high as 50% in borderline CH cases with blood spot TSH of 6–20 mU/L (36). In our cohort, while monoallelic *DUOX2/DUOX2A2* variants were found only in two patients with PCH as a part of oligogenicity, they were causative in 22% of the TCH cohort.

An interesting case of TCH (#3) in our cohort had homozygous *SLC26A4* and heterozygous *SLC5A5* variants. Consanguineous parents with normal thyroid function were heterozygous for *SLC26A4* variant. Thyroid imaging and hearing tests were normal in the newborn and at re-evaluation. Therefore, the patient had no symptoms consistent with Pendred syndrome and stayed at follow-up for possible progress to hearing loss and goiter, and for recurrence of hypothyroidism. To our knowledge, *SLC26A4* gene defects associated with TCH have not been reported to date. The phenotypic spectrum of thyroid in the biallelic *SLC26A4* mutations is very complex and extends from normal function and morphology to acquired subclinical or overt hypothyroidism, as well as CH with usually goiter (37) but very rarely with thyroid

hypoplasia (38). Moreover, the thyroid phenotype is modified by nutritional iodide intake. Development of goiter and hypothyroidism in *SLC26A4* gene defects is uncommon under conditions of adequate or high iodine intake, whereas it is more prevalent in the state of iodine deficiency (39). Insufficient iodine intake is a continuing problem in pregnant women and their offspring in Turkey (40, 41, 42, 43). Thus, we hypothesized that with mutations in *SLC26A4* and *SLC5A5* involved in iodine transport in the thyroid gland, case #3 might have presented as hypothyroidism at birth with the possible contribution of iodine deficiency and recovered over time under LT4 therapy. Concluding that this is a representative case of TCH arising from the interaction of genetic and environmental factors, we considered these oligogenic variants causative. Consequently, our study disclosed the presence of genetic contribution in about one-quarter of TCH cases treated for more than 2 years and thereby provided additional evidence for the predominant role of environmental factors in the etiopathogenesis of TCH in our population.

Although our data reveal the importance of genetics in the management of patients with CH, the main determinant in the treatment decision is of course thyroid function tests, which reflect the state of the hypothalamic–pituitary–thyroid (HPT) axis. The HPT axis is finely tuned to maintain a stable concentration of FT4 within any individual. A downward deviation from this individual set point due to thyroid dysfunction results in an increase in TSH concentration even if FT4 remains within the reference range, which justifies the definition of subclinical hypothyroidism. An exception to this is pituitary thyroid hormone resistance. In this state, the relatively high TSH concentration is an appropriate response of the hypothalamic–pituitary axis with reduced sensitivity to thyroid hormone (44). Likewise, elevated TSH levels in individuals with TSH resistance reflect an appropriately adjusted set point for the HPT negative feedback control axis (5). Therefore, in the presence of hormone resistance in the HPT axis, high TSH secretion does not necessarily indicate subclinical hypothyroidism that requires treatment.

The need for treatment in children with subclinical hypothyroidism has been a matter of debate, but it is still recommended that children with moderately elevated TSH levels (>10 mU/L) be treated for subtle cardiovascular dysfunction and proatherogenic abnormalities (45). There is still controversy about the necessity of replacement therapy in children with isolated hyperthyrotropinemia resulting from TSH resistance (46). However, studies in the

past decade have shown that untreated children with well-compensated hyperthyrotropinemia due to heterozygous *TSHR* mutations have normal growth and development (5, 6, 47). So, we chose to discontinue LT4 therapy in two such 9-year-old children (cases #7 and #46) with their parents' informed consent. Nonetheless, even if we knew the monoallelic *TSHR* variants in the newborn period, we would not dare to leave these children untreated, whose TSH levels ranged from 15 to 88 mU/L, and FT4 levels were close to the lower limit of the reference ranges. Given other possible genetic defects not covered by the HTS panel and other environmental factors that may affect the HPT axis, LT4 treatment would be a more reasonable approach during the first 3 years of life when most thyroxine-dependent brain maturation has occurred.

Likewise, treatment was mandatory for overt CH with low FT4 levels in case #7 with two monoallelic variants in *TSHR* and *DUOXA2* genes. In fact, the treatment decision is made according to the thyroid hormone profile, not the mutations. But, the recognition of a *DUOXA2* defect during early infancy provides an insight that CH may be transient (36). Also, awareness of TSH resistance contributes to a more accurate interpretation of the HPT axis, as discussed above. As a matter of fact, in this interesting case of oligogenic involvement of *DUOXA2* and *TSHR*, severe hypothyroidism resolved in 2 years, but mild isolated hyperthyrotropinemia persisted thereafter. Finally, the genetic analysis provided an explanation supporting the decision to follow up without treatment for mildly elevated TSH in childhood.

Our study has some potential limitations and strong points. This is a hospital-based, regional study limited to 48 cases. Additional studies in larger cohorts are needed to confirm our data on the genetics of CH. Another limitation is that the pathogenicity of the variants identified in our study was solely analyzed *in silico*. Functional analysis is particularly essential for 15 novel variants, which were described for the first time in this study. On the other hand, one of the strengths of our study is that compared to previous studies, the genetic data obtained for the research has been used in the management of children with CH in daily practice. In this way, our study has shown that genetic testing had positive effects on the diagnosis, treatment and prognosis of CH patients. In addition, this study has revealed for the first time the relative contribution of genetic causes to the prevalence of TCH in our population.

In summary, the targeted 23-gene HTS panel yielded a definitive molecular diagnosis in 57% of PCH patients with GIS. However, the genetic origin of TCH was found to be

relatively low, suggesting that environmental factors may have a predominant role in the etiopathogenesis of TCH. The most common genetic causes of GIS-CH in our cohort were mono- and biallelic *TG*, *TSHR* and *DUOXA2* variants. Re-evaluation based on genetic testing allowed us to cease treatment in five patients, representing about 10% of the GIS-CH cohort. Consequently, genetic testing can change diagnosis and treatment decisions in a small proportion of children with CH, but the resulting benefit may outweigh the burden of lifelong follow-up and treatment. These data need to be confirmed in larger patient groups. Also, *in vitro* functional studies are required to verify the pathogenicity of novel variants described for the first time in this study.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/ETJ-22-0212>.

Declaration of interest

The authors have nothing to disclose.

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Author contribution statement

C K, J M, A D and M A designed the study. C K, J M, E İ G, and M A recruited and clinically characterized the patients. Ü A and C K acquired funding. C G performed high-throughput and Sanger sequencing. C G, C K and Ü A carried out bioinformatics analysis of the identified variants. C K and J M prepared the draft manuscript. All authors contributed to the discussion of results, and edited and approved the final manuscript.

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