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Original Article

# Clinical and genetic insights into congenital hypothyroidism with gland-in-situ

Fatma Dursun[1], Metin Eser[2], Murat Hakkı Yarar[2], Irmak Dicle Sargın[1], Ayşe Yaşar[1], Esma Ebru Altun[1], Fatma Güliz Atmaca[1], Şerife Ece Ulu[1], Heves Kırmızıbekmez[1]

[1]Department of Pediatric Endocrinology, University of Health Sciences Ümraniye Training and Research Hospital, İstanbul, Türkiye
[2]Department of Medical Genetics, University of Health Sciences Ümraniye Training and Research Hospital, İstanbul, Türkiye

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## ABSTRACT

**Objective:** This study aimed to investigate the genetic etiology of congenital hypothyroidism (CH) with gland-in-situ (GIS) by screening 23 candidate genes using next-generation sequencing (NGS) in a cohort of Turkish patients.

**Methods:** A total of 44 patients diagnosed with GIS-CH were enrolled. Genetic analysis was performed using a targeted gene panel including 23 genes related to thyroid development and function. Clinical and demographic features were compared between permanent CH (PCH) and transient CH (TCH) groups. Patients with thyroid dysgenesis, central hypothyroidism, syndromic features, or insufficient follow-up were excluded.

**Results:** Genetic variants were identified in 43.1% (19/44) of patients, with similar frequencies in PCH (46.1%) and TCH (38.8%) groups. Ultrasonography revealed hyperplasia in two patients, hypoplasia in 10, and normal-sized gland-in-situ (GIS) in 32 patients. The most frequently identified variant was in the TSHR gene (15.9%), followed by DUOX2 (11.3%), PAX8 (6.8%), TG (4.5%), and DUOXA2 (2.2%). Homozygous or compound heterozygous variants were consistently associated with PCH. In contrast, heterozygous variants showed variable clinical outcomes, ranging from transient disease to persistent hypothyroidism requiring ongoing L-thyroxine therapy. An oligogenic variant (DUOX2+TG) was identified in one patient and was associated with a milder clinical course. Thyroid hypoplasia was predominantly observed in patients with TSHR and PAX8 variants and was strongly associated with the need for continued treatment, even in heterozygous cases.

**Conclusions:** The most common genetic causes of GIS-CH in our cohort were mono- and biallelic TSHR, DUOX2, and monoallelic PAX8 variants. Thyroid hypoplasia was observed predominantly in patients with TSHR and PAX8 variants and was consistently associated with the continued requirement for L-thyroxine therapy, even among heterozygous cases. The main limitations of this study include the relatively small sample size and lack of functional validation of identified variants.

**Keywords:** transient, permanent, congenital hypothyroidism, gland-in-situ

## INTRODUCTION

Congenital hypothyroidism (CH) is the most prevalent endocrine disorder in neonates and has historically been attributed predominantly to thyroid dysgenesis, which was estimated to account for approximately 80% of cases.[1] Based on pathophysiological classification, most cases of CH are categorized as either thyroid dysgenesis (TD) or dyshormonogenesis (DH). However, recent studies indicate that the apparent rise in the incidence of CH, currently estimated at approximately 1 in 1500 live births, is largely due to increased detection of CH cases with eutopic glands in situ (GIS).

✉ Fatma Dursun ▪ fatmadursun54@yahoo.com

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Accordingly, the proportion of patients with GIS has increased to about 35–40%, and in some studies, CH with a eutopic thyroid gland accounts for nearly two-thirds of newly diagnosed cases.[2,3] Possible explanations for the increased frequency of GIS-CH include lower TSH screening thresholds, increased functional thyroid defects due to iodine deficiency or excess, exposure to thyroid-disrupting chemicals, and a higher incidence of premature births.[4] In addition, as molecular studies accelerate, new genes and mechanisms are being discovered, and the etiology of CH appears to be more complex than previously thought.[5] It has previously been estimated that the genetic variants in the candidate genes for both TD and DH can be detected in approximately 20% of patients with CH.[6,7] Recent studies have reported an increased rate of variant detection with the application of next-generation sequencing (NGS); however, findings have varied across studies, and the pathogenic significance of some identified variants remains unclear.[8,9] Nevertheless, NGS has been demonstrated to be both cost-effective and robust, thus indicating that traditionally goiter-associated candidate genes, such as DUOX2 and thyroid peroxidase (TPO), may also be implicated in TD. Consequently, these findings suggest an increasingly complex genotype-phenotype relationship in CH.[10,11] The increasing identification of genetic etiology in CH is attributed to several factors, including the widespread use of advanced genetic analysis methods such as NGS, the diagnosis of a greater number of patients through neonatal CH screening programs, and the higher contribution of genetic factors, particularly in patients with GIS. With advances in technology, it has become evident that the genetic basis of CH is highly complex.[5]

In this study, the aim was to screen 23 candidate genes using NGS in a cohort of Turkish children diagnosed with gland in situ CH.

## MATERIALS AND METHODS

The study was conducted on patients who were diagnosed with CH and started treatment in the pediatric endocrinology clinic. Between June 2024 and June 2025, 175 patients with CH who were regularly followed in the pediatric endocrinology outpatient clinic were reviewed. Among these patients, 59 with CH and GIS who underwent genetic analysis were identified. Of these, 44 patients with complete clinical and genetic data were included in the study. Patients with an identified thyroid dysgenesis (agenesis, hemiagenesis, or ectopy), patients with syndromes like Down syndrome, patients with central hypothyroidism, and patients with thyroid dysfunction due to prematurity or maternal antithyroid drugs were excluded from the study. Furthermore, patients with transient hypothyroidism who required less than two years of treatment and had not yet reached the adequate follow-up period of two years were excluded from the study.

The study was performed according to the Helsinki II declaration and approved by the local Ethical Committee of our hospital (approval number: 20/05/2024-244120985).

The national screening program for CH has been implemented in Turkey since 2006. Dry blood samples are collected between days 2 and 5, and infants with TSH levels >5.5 µU/mL are referred to hospitals for serum TSH and free thyroxine (FT4) levels.[12] Primary hypothyroidism was diagnosed with elevated TSH and low/normal FT4 levels, using reference values. Thyroid function tests were measured in the early morning fasting blood samples using the chemiluminescent immunometric method (Architect i4000, Abbott Laboratories Diagnosis Division, IL, USA) in the biochemistry laboratory of our hospital, where the reference range for serum TSH concentrations was 0.69-5.8 mIU/L and for FT4 was 0.85-1.7 ng/dL.

All patients in this study with low serum FT4 and/or persistently elevated TSH levels (>10.0 µU/mL) were treated with L-thyroxine (LT4).

The study was designed as a retrospective analysis based on a review of patients' medical records and follow-up data, including treatment requirements beyond 2 years. The patients were initially classified into two diagnostic categories-Permanent Congenital Hypothyroidism (PCH) and Transient Congenital Hypothyroidism (TCH)-based on findings at presentation, throughout follow-up, or upon re-evaluation after the age of 2–3 years. PCH was defined as the necessity to augment the dosage of LT4 in cases of elevated TSH levels on subsequent follow-ups and the requirement to reinitiate medication in instances where TSH levels exceeded 10 µU/mL following the cessation of treatment. TCH was defined as initiation of LT4 treatment during the neonatal period, followed by discontinuation during the subsequent follow-up period, without the need to restart treatment. This definition was applied to infants in whom FT4 was normal and TSH was normal or <10 µU/mL six months after the cessation of treatment. Transient cases requiring at least two years of LT4 treatment were included in the genetic analyses, given the predominance of environmental factors in the etiology of this group.[13-17]

A comparative analysis was conducted of permanent CH (PCH) and transient CH (TCH) patients with respect to demographic characteristics and genetic variants.

Thyroid ultrasonography (USG) was performed by an experienced pediatric radiologist. All examinations were conducted with patients in the supine position and the neck in slight hyperextension. High-resolution linear transducers with frequencies of 4.8–11 MHz or 5–14 MHz were used (Toshiba Aplio 500, Aplio 300; Toshiba Medical Systems, Tokyo, Japan; Siemens Acuson S3000, Siemens Medical Solutions, USA). Both transverse and longitudinal scans were obtained using real-time grayscale and color Doppler imaging. Thyroid volume was calculated using the formula (Thyroid volume = depth × length × width × 0.479), with age-matched references for Turkish children. Standard deviation scores (SDS) were computed using the CHILD METRICS software developed by the Pediatric Endocrine and Diabetes Association.[18] Glands with SDS values below −2 were classified as hypoplastic, between −2 and +2 as normal, and above +2 as hyperplastic. In this study, GIS was defined as the presence of a eutopic thyroid gland located in its normal anatomical position on ultrasonography, regardless of gland size. Thus, GIS included all patients with a normally located thyroid gland, irrespective of size variations. Patients with thyroid agenesis and ectopic thyroid were excluded from the study.

### Genetic analysis

Peripheral blood samples were obtained from all participants after written informed consent was secured. Genomic DNA was isolated from EDTA-anticoagulated whole blood using automated extraction systems (Qiagen), following the manufacturer's protocols. DNA concentration and purity were evaluated using Qubit fluorometry and UV spectrophotometry (260/280 and 260/230 ratios). Library preparation for next-generation sequencing (NGS) was performed using the Sophia Genetics Inherited Disease Panel, including a custom 23-gene panel related to congenital hypothyroidism (DUOX2, DUOXA2, GABRD, GLIS3, IYD, KCNAB2, KDM6A, KMT2D, NKX2-1, NKX2-5, PAX8, PDE4D, PRDM16, PRKAR1A, SKI, SLC26A4, SLC5A5, TG, THRA, THRB, TPO, TSHB, TSHR). The sequencing was carried out on the Illumina NextSeq 500 platform.

### Bioinformatic and variant interpretation

Raw sequencing data were analyzed using the Sophia DDM® platform. Sequence alignment and variant calling were performed via the proprietary Pepper® algorithm, based on the GRCh37/hg19 reference genome. Variant annotation was conducted using MOKA® software and included data on mutation type (e.g., missense, nonsense), population frequency (from 1000 Genomes, gnomAD, ExAC), and in silico predictions (e.g., SIFT, PolyPhen). Mean sequencing coverage was approximately 90% at an average depth of 30×. A minimum read depth threshold of 30 reads was applied to ensure reliable variant calling. Variants were filtered using a variant allele fraction (VAF) cutoff of ≥15%, thereby prioritizing those with sufficient representation for downstream analysis.

Copy number variation (CNV) analysis was conducted using Sophia's MUSKAT® software. Variant pathogenicity was interpreted according to the American College of Medical Genetics and Genomics (ACMG) guidelines, incorporating ClinVar annotations and published evidence, including criteria proposed by Maxwell et al. For evidence codes such as PP2, BP1, and PVS1, gene-specific mechanisms and functional consequences were considered. Rare variants were filtered using a minor allele frequency (MAF) threshold of <0.0001 for PM2 classification.[19-27]

### Statistical analysis

All statistical analyses were performed using SPSS Statistics version 26.0 (IBM Corp., Armonk, NY, USA). Normality of continuous variables was assessed using the Kolmogorov–Smirnov test. Descriptive data are presented as mean ± standard deviation (SD) for normally distributed variables and as median (interquartile range, IQR) for non-normally distributed variables. Between-group comparisons were conducted using the Student's t-test or Mann–Whitney U test, as appropriate. Categorical variables were compared using the Chi-square test. A p-value of <0.05 was considered statistically significant.

## RESULTS

In this study of 44 patients (23 female, 21 male) diagnosed with CH, 18 had TCH and 26 had PCH. The mean ages of patients with TCH and PCH were 22.5 ± 13 and 20 ± 19 days, respectively. Parental consanguinity was present in 23.3% of the patients. All but two siblings were from different families. The prevalence of hypothyroidism in the families of both groups did not exhibit a statistically significant difference (61.5% in PCH, 38.5% in TCH, p=0.68). Ultrasonography revealed hyperplasia in two patients, hypoplasia in 10, and normal-sized gland-in-situ (GIS) in 32 patients. Genetic variants were detected in 19 of 44

patients (43.1%), with no significant difference between the two groups (p=0.86), as detailed in Table 1.

**Genetic results**

Among the 19 patients with detected genetic variants, nine were homozygous and nine were heterozygous. One patient had an oligogenic variant (DUOX2+TG). In total, 17 different variants were identified in 18 families (Table 2). Two siblings from one family were found to have a homozygous TSHR variant (c.1349G>A p. Arg450His). The most frequently affected gene was TSHR (15.9%), followed by DUOX2 (11.3%), PAX8 (6.8%), TG (4.5%), and DUOXA2 (2.2%), respectively. In addition, one patient was found to have an oligogenic variant involving DUOX2 and TG. All patients with homozygous genetic variants were diagnosed with PCH, whereas the clinical diagnosis of heterozygous variant carriers was variable. Two of the three patients with TSHR heterozygous variants had PCH, while one of the three patients with PAX8 heterozygous variants had PCH. All three patients with PCH due to heterozygous variants had hypoplastic thyroid glands (TSHR heterozygous variant in two patients and PAX8 heterozygous variant in one patient).

Based on long-term clinical follow-up, biochemical findings, and the absence of hypothyroidism after supervised LT4 withdrawal, treatment was discontinued and not restarted in six patients (one with TSHR heterozygosity, one with DUOX2 heterozygosity, one with DUOX2+TG oligogenic disease, one with DUOXA2 heterozygosity, and two with PAX8 heterozygosity), rather than solely on genetic results (Table 3).

**TSHR variants**

All patients with homozygous variants in the TSHR gene had PCH. Their thyroid volumes were compatible with hypoplasia. Treatment for three patients with heterozygous variants in the TSHR gene was discontinued after the genetic results; however, it was restarted for two of them (Table 3). After cessation of LT4, one had a TSH level of 27 mIU/L and a concomitant secondary amenorrhea, which improved after normalization. The other had no symptoms, but treatment was restarted because her TSH level had risen to 25 mIU/L. Both had hypoplasia of the thyroid gland.

**DUOX2 variants**

Four patients harbored homozygous or compound heterozygous DUOX2 variants and were diagnosed with PCH. Thyroid USG revealed hyperplasia in two patients, while thyroid volumes were normal in the other two (Table 3). One patient developed a nodule in the thyroid gland (patient 42) and underwent a total thyroidectomy with a fine-needle aspiration biopsy (FNAB) result of atypia of unknown etiology (AUS) (Bethesda III). The pathology result was interpreted as benign. In the other three patients, iodine treatment was initiated in conjunction with L-thyroxine therapy after the genetic result. A heterozygous

**Table 1.** Clinical features and laboratory findings of patients with transient and permanent congenital hypothyroidism at diagnosis and at follow-up

| Parameters | Transient n=18 | Permanent n=26 | p |
|---|---|---|---|
| Gender: F/M (n) | 10/8 | 13/13 | 0.950[a] |
| Age at diagnosis (day) | 22.5±13 | 20±19 | 0.660[b] |
| Gestational age (week) | 40 (29-42) | 40 (29-42) | 0.200[c] |
| Consanguinity (%) | 11 | 32 | 0.110[a] |
| Birth weight (g) | 2845±744 | 3145±756 | 0.210[b] |
| Family history for CH (%) | 38.5 | 61.5 | 0.680[a] |
| First TSH (NR: 0.69-5.8mIU/L) | 55 (7-216) | 91 (13-500) | *0.050[c] |
| First FT4 (NR: 0.85-1.7 ng/dL) | 0.9±0.29 | 0.64±0.28 | *0.050[b] |
| Thyroid volume SDS | -0.89 (-1.9-1.3) | -0.43 (-2.8-2.7) | 0.370[c] |
| LT4 dose at diagnosis (µg/kg/day) | 8.7±2.7 | 9.7±3.3 | 0.380[b] |
| LT4 dose at 24 months (µg/kg/day) | 1.6±0.43 | 2.9±0.9 | *0.010[b] |
| Height at last visit (SDS) | -0.54±0.9 | -0.19±0.1 | 0.270[b] |
| Genetic etiology: yes/no (n) | 7/11 | 12/14 | 0.860[a] |

F, female; M, male; CH, congenital hypothyroidism; TSH, thyroid-stimulating hormone; FT4, free thyroxine; NR, normal range; SDS, standard deviation score; L-T4, L-thyroxine; n, number. *p≤0.05, a: Chi-square test, b: Independent samples T test, c: Mann Whitney U test.

**Table 2.** The variants and proteins in the genes associated with congenital hypothyroidism

| Case | CF | Gene | Zygosity | Variant | Protein | ACMG classification |
|---|---|---|---|---|---|---|
| #9 | yes | TSHR | HT | c.484C>G | Pro162Ala | VUS (PM2, PP3) |
| #10 | No | TSHR | HM | c.1422 C>A | Asp474Glu | VUS (PM2, PP3) |
| #13 | No | TSHR | HT | c.1422C>A | Asp474Glu | VUS (PM2, PP3) |
| #15 | Yes | TSHR | HM | c.434C>T | Thr145Ile | VUS (PM2, PP3) |
| #21 | Yes | TSHR | HM | c.1349G>A | Arg450His | VUS (PM2, PP3, PP5) |
| #22 | Yes | TSHR | HM | c.1349G>A | Arg450His | VUS (PM2, PP3, PP5) |
| #44 | No | TSHR | HT | c. 351A>G | Ile117Met | VUS (PM2, PP3) |
| #23 | Yes | DUOX2 | HT | c.1464 del | Leu489Serfs*97 | Likely Pathogenic (PVS1, PM2) |
| #27 | Yes | DUOX2 | HM | c.3416-2A>C | splice acceptor | Likely Pathogenic (PVS1, PM2 |
| #41 | Yes | DUOX2 | CHT | c.2895_2898del<br>c.3968C>A | Phe966Serfs*29<br>(Ala1323Glu) | Pathogenic (PVS1, PM2, PP5)<br>VUS (PM2, PP3) |
| #42 | Yes | DUOX2 | HM | c.1300 C>T | Arg434* | Pathogenic (PVS1, PM2, PP5) |
| #43 | No | DUOX2 | HM | c. 3251G>A | Arg1084Gln | Likely Pathogenic (PM1, PP3) |
| #6 | No | PAX8 | HT | c.1109C>T | Thr370Met | VUS (PM2, PP3) |
| #37 | No | PAX8 | HT | c.97C>T | Arg33Cys | VUS (PM2, PP3) |
| #39 | No | PAX8 | HT | c.161G>A | Ser54Asn | VUS (PM2, PM5, PP3) |
| #30 | Yes | TG | HM | c.886C>T | Arg296* | Pathogenic (PVS1, PM2, PP5) |
| #32 | No | TG | HT | c.886C>T | Arg296* | Pathogenic (PVS1, PM2, PP5) |
| #26 | No | DUOX2+TG | HT/HT | c.2048G>A/<br>c. 3149G>T | Arg683His/Trp1050Leu | VUS (PM2)<br>VUS (PP3) |
| #8 | No | DUOXA2 | HT | c.33C>A | (Tyr11*) | Likely Pathogenic (PVS1, PM2) |

HT, heterozygous; HM, homozygous; CHT, compound heterozygous; CF, consanguineous family; F, female; M, male; *, stop codon

variant in DUOX2 was identified in one patient, and a DUOX2+TG oligogenic variant was detected in one patient (Table 3). After genetic results, L-thyroxine treatment was discontinued in both patients. The TSH level increased to 14 mIU/L in the patient with the DUOX2 variant; treatment was not restarted, and no symptoms were observed. In the oligogenic patient, TSH levels remained within normal limits following the cessation of treatment.

After the detection of a heterozygous variant in DUOXA2 in one patient, L-thyroxine replacement was withdrawn following variant interpretation.

### PAX8 variants

A heterozygous PAX8 variant was detected in three patients (Table 2). Treatment was discontinued in two patients after genetic analysis, while treatment was continued in one patient (patient 39). The result of this patient showed a heterozygous c.161G>A (p.Ser54Asn) variant, but the thyroid was hypoplastic (TV SD: -2.1 SD). At the age of 2 years, this patient was able to maintain normal TSH levels with an L-T4 dose of 5 mcg/kg/day.

### Thyroglobulin variants

A homozygous variant in the TG gene was found in one patient and a heterozygous variant in one patient (Table 2). While treatment of the heterozygous patient could be discontinued before the age of 2 years, the homozygous patient had multiple admissions with very high TSH levels (TSH >200 mIU/mL, FT4 <0.5 ng/dL).

## DISCUSSION

Using next-generation sequencing (NGS), we screened 23 candidate genes in 44 Turkish CH patients. Genetic etiology was identified in 19 patients (43.1%); 7 of 18 patients (38.8%) with TCH and 12 of 26 patients (46.1%) with PCH. Among the 19 patients with variants, nine were heterozygous, nine homozygous or compound heterozygous, and one had an oligogenic variant (DUOX2+TG). The most frequently identified variant was in the TSHR gene (15.9%), followed by DUOX2 (11.3%), PAX8 (6.8%), TG (4.5%), and DUOXA2 (2.2%). Targeted candidate-gene analysis using NGS for CH has been reported with increasing frequency. However,

**Table 3.** Phenotypic features and thyroid dysfunction status in patients with identified genetic variations associated with congenital hypothyroidism

| Case | Gender | Gene | Zygosity | Thyroid Volume (SDS) | Structure | Treatment | Age at last visit (year) | LT4 dose µg/kg/d | Last TSH mIU/L | Last FT4 ng/dL |
|---|---|---|---|---|---|---|---|---|---|---|
| #9 | F | TSHR | HT | -1.96 | Hypoplasia | Yes | 5.5 | 1.8 | 27 [a]<br>4 [b] | 1.0 [a]<br>1.6 [b] |
| #10 | F | TSHR | HM | -2.01 | Hypoplasia | Yes | 11 | 3.1 | 1.4 | 0.97 |
| #13 | F | TSHR | HT | -1.81 | Normal | No* | 8.5 | - | 8.9 | 1.3 |
| #15 | F | TSHR | HM | -1.98 | Hypoplasia | Yes | 9.0 | 2.0 | 5.1 | 1.4 |
| #21 | F | TSHR | HM | -2.01 | Hypoplasia | Yes | 11.0 | 3.0 | 23 | 0.8 |
| #22 | M | TSHR | HM | -1.99 | Hypoplasia | Yes | 8.8 | 5.0 | 6.5 | 1.1 |
| #44 | F | TSHR | HT | -2.81 | Hypoplasia | Yes | 17 | 1.1 | 25 [a]<br>6.8 [b] | 0.9 [a]<br>1.1 [b] |
| #23 | M | DUOX2 | HT | -1.48 | Normal | No* | 12 | - | 14.5 | 1.2 |
| #27 | M | DUOX2 | HM | 1.44 | Normal | Yes | 5.8 | 1.8 | 2.4 | 1.5 |
| #41 | F | DUOX2 | CHT | 2.50 | Hyperplasia | Yes | 11.5 | 2.0 | 3.8 | 1.4 |
| #42 | M | DUOX2 | HM | 2.71 | Hyperplasia | Yes | 8.5 | 3.0 | 4.2 | 1.5 |
| #43 | F | DUOX2 | HM | -0.37 | Normal | Yes | 5.2 | 3.1 | 6.5 | 1.3 |
| #6 | M | PAX8 | HT | -0.61 | Normal | No* | 14.0 | - | 6.08 | 1.4 |
| #37 | M | PAX8 | HT | -1.12 | Normal | No * | 6.0 | - | 5.6 | 1.2 |
| #39 | M | PAX8 | HT | -2.11 | Hypoplasia | Yes | 2.0 | 5.0 | 21 [a]<br>4.5 [b] | 1.6 |
| #30 | M | TG | HM | 1.67 | Normal | Yes | 13.5 | 2.0 | 16 | 1.7 |
| #32 | F | TG | HT | 0.46 | Normal | No* | 3.5 | - | 8.0 | 1.1 |
| #26 | M | DUOX2+TG | HT/HT | 1.30 | Normal | No* | 5.5 | - | 4.1 | 1.2 |
| #8 | F | DUOXA2 | HT | -0.76 | Normal | No* | 4.8 | - | 9.1 | 1.2 |

HT, heterozygous; HM, homozygous; CHT, compound heterozygous; F, female; M, male; TSH, thyroid-stimulating hormone; FT4, free thyroxine; SDS, standard deviation score; y, year; d, day

* Discontinued after genetic analysis,

a: TSH and FT4 levels measured after the discontinuation of L-thyroxine therapy; b: TSH and FT4 levels measured during ongoing L-thyroxine treatment.

detection rates vary widely due to differences in patient selection, variant filtering strategies, and pathogenicity assessment. Recent studies have identified genetic variants in 33.0% to 51.5% of CH cases, and the results of the present study are consistent with these previously reported ranges.[28-32]

Yamaguchi et al.[7], in a study of 167 patients including dyshormonogenesis (57 patients), dysgenesis (32 patients), and CH patients of unknown etiology (78 patients), detected 66.5% variants by NGS. Of these variants, 2.6% were biallelic, 18% were oligogenic, and 35.9% were monoallelic. Kara et al.[13] studied 48 patients with CH, and a genetic etiology was found in 65% of patients, which was higher than in our study. This difference can be attributed to the selection of GIS cases in our study. PAX8 mutations are typically inherited in an autosomal dominant (AD) manner, and autosomal recessive cases have not been reported to date. These AD variants are generally associated with PCH.[17] A single case of TCH has been reported in association with a heterozygous missense mutation in PAX8, a key thyroid transcription factor essential for both thyroid morphogenesis and the maintenance of normal thyroid structure and function in adulthood.[33] Consistent with the case reported in the literature, treatment was successfully discontinued in two of the three patients harboring PAX8 variants in our cohort. One patient presented with thyroid hypoplasia, and two patients had normal thyroid volume with congenital hypothyroidism. These findings suggest that additional, yet unidentified epigenetic, environmental or unidentified factors may contribute to the phenotypic variability observed in TCH or PCH beyond genetic mutations.

Previous studies have shown that the mutation detection rate in cases of thyroid dysgenesis (TD) is approximately 5%, mainly involving the PAX8 and TSHR genes.[17,34] However, TSHR and PAX8 variants, which may present with either thyroid hypoplasia or normal thyroid volume, can be observed in both thyroid dysgenesis and gland-in-situ (GIS). Therefore, the identification of TSHR or PAX8 variants in patients with GIS is not unexpected.[35] In the present cohort, variants were most frequently identified in TSHR (7/19), followed by DUOX2 (5/19) and PAX8 (3/19). Yamaguchi et al.[7] found variants most frequently in DUOX2, followed by TG. In the study by Kara C et al from Turkey[13], the most common variants were in the TG, TSHR, and DUOX2. In the study by Nicholas et al.[2] in CH patients with GIS, the most frequent variants were in TG, followed by TPO, whereas DUOX2 variations were relatively rare compared to the previous literature. DUOX2 mutations appear to be more common in individuals of Asian origin.[2]

Thyroid glands tend to be hypoplastic in patients with TSHR variants All homozygous TSHR variants were found to be associated with persistent hypothyroidism, while heterozygous TSHR variants tended to require treatment when the patient had hypoplastic Tenenbaum-Rakover et al.[36] showed that in individuals heterozygous for loss-of-function TSHR mutations represent a stable, compensated state, which does not necessitate replacement therapy. However, there were no patients with thyroid hypoplasia in their study, while some patients did not even have US findings. Treatment cessation could not be made in one of three patients with a PAX8 variant (patient 39), who had a heterozygous variant and a hypoplastic thyroid gland. By the age of two, this patient could maintain normal TSH levels with an average L-T4 dose of 5 mcg/kg/day. Stoupa et al.[35] stated that thyroid morphology and the severity of biochemical hypothyroidism exhibit considerable variability in PAX8 variants, with cases ranging from severe congenital hypothyroidism to mild hyperthyrotropinemia observed even within the same family.

Reassessment is deemed unnecessary for patients with CH presenting with ectopic thyroid or athyreosis. However, current guidelines provide no definitive recommendations regarding reassessment in cases of thyroid hypoplasia.[14] Rabbiosi et al. have shown that thyroid hypoplasia on US was correlated with PCH, and that no patients with hypoplastic thyroid harbored TCH.[16] Our study showed that heterozygous variants can cause hypoplastic gland and suggested that although the variant is heterozygous, having a hypoplastic thyroid gland is more important in PCH-TCH differentiation.

In our study, when TCH and PCH were compared in terms of demographic characteristics, TSH levels at presentation were significantly higher in PCH patients. Among the studies in literature, Scavone et al. also found a significantly different baseline TSH levels of 24.2 (52.4) uIU/L in TCH patients and 73.3 (276.5) uIU/L in PCH patients (p:0.013).[37]

The prevalence of DUOX2 mutations among CH patients is highly variable and generally high, with 44% in the Netherlands[38], 43% in Japan[6], 30-45% in Italy[39] and 29-83% in China.[40] While this difference may be attributed to differences in the methodologies of the studies, genetic diversity among different ethnic groups may also play a contributing role. In the current study, we identified six patients with DUOX2 variation, four with homozygous or compound heterozygous variants, and two with heterozygous variants. Those homozygous for DUOX2 were observed to have PCH, whereas those heterozygous for DUOX2 were observed to have TCH or mild subclinical hypothyroidism, which did not require treatment.

### Study limitations

The primary limitation of our study was its hospital-based, regional design, which resulted in a relatively small sample size. To validate our findings on the genetics of CH, additional studies with larger cohorts are necessary. Another notable limitation was the exclusive in silico analysis of the pathogenicity of the variants, which lacks experimental validation. Another limitation of this study is that patients with thyroid agenesis were not included; therefore, the phenotypic spectrum associated with severe PAX8 mutations may not be fully represented. A major limitation of this study is the exclusion of patients with insufficient follow-up and the selective application of genetic testing, which may have influenced variant detection rates and the observed phenotype distribution.

## CONCLUSION

In this study, the most common genetic causes of GIS-CH in our cohort were mono- and biallelic TSHR, DUOX2, and monoallelic PAX8 variants. While homozygous mutations cause PCH, we found that the clinical presentation of heterozygous mutations can be very variable. We found that heterozygous TSHR and PAX8 variants may require treatment at a higher rate compared to studies in the literature. We observed that the need for treatment persists when thyroid hypoplasia is present in heterozygous variants. Thyroid volume appears to be an important criterion for the persistent need for thyroid hormone replacement therapy

in patients with heterozygous variants, which needs to be confirmed in larger patient groups.

## Author contributions

Conception and design: F.D., M.E.; Data acquisition: A.Y., E.E.A., Ş.E.U., F.G.A., İ.D.S.; Data analysis: F.D., M.E., M.H.Y.; Data interpretation: F.D., M.E., M.H.Y.; Drafting of the manuscript: F.D., M.E., H.K. All authors reviewed the results, approved the final version of the manuscript, and agreed to be accountable for all aspects of this study.

## Ethical approval

This study was approved by the local Ethical Committee of University of Health Sciences, Umraniye Training and Research Hospital (Decision/Protocol No: 20/05/2024-244120985). Informed consent was obtained from all participants involved in this study.

## Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## Conflict of interest

The authors declare that this study was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Generative AI statement

The authors declare that no generative AI or AI-assisted technologies were used in the writing or preparation of this study.

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