

Detection of Novel Gene Variants Associated with Congenital Hypothyroidism in a Finnish Patient Cohort

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Background: Congenital hypothyroidism (CH) is defined as the lack of thyroid hormones at birth. Mutations in at least 15 different genes have been associated with this disease. While up to 20% of CH cases are hereditary, the majority of cases are sporadic with unknown etiology. Apart from a monogenic pattern of inheritance, multigenic mechanisms have been suggested to play a role in CH. The genetics of CH has not been studied in Finland so far. Therefore, multigenic sequencing of CH candidate genes was performed in a Finnish patient cohort with both familial and sporadic CH.

Methods: A targeted next-generation sequencing (NGS) panel, covering all exons of the major CH genes, was applied for 15 patients with sporadic and 11 index cases with familial CH.

Results: Among the familial cases, six pathogenic mutations were found in the *TPO*, *PAX8*, and *TSHR* genes. Furthermore, pathogenic *NKX2.1* and *TG* mutations were identified from sporadic cases, together with likely pathogenic variants in the *TG*, *NKX2.5*, *SLC26A4*, and *DUOX2* genes. All identified novel pathogenic mutations were confirmed by Sanger-sequencing and characterized *in silico* and/or *in vitro*.

Conclusion: In summary, the CH panel provides an efficient, cost-effective, and multigenic screening tool for both known and novel CH gene mutations. Hence, it may be a useful method to identify accurately the genetic etiology for dysmorphogenic, familial, or syndromic forms of CH.

Introduction

PRIMARY CONGENITAL HYPOTHYROIDISM (CH) is a relatively common endocrine disease affecting 1 in 2000–4000 newborns worldwide (1–3). It is defined as the lack of thyroid hormones at birth. If not identified and treated promptly, it can lead to severe developmental delay and mental retardation (4). In addition, patients with syndromic CH often have other congenital malformations such as lung, kidney, or heart defects (5). Therefore, in many countries,

newborn children are screened for CH by measuring serum thyrotropin (TSH) and/or thyroxine (T4).

The majority (80%) of primary CH is a consequence of thyroid dysgenesis (TD)—developmental abnormalities of the thyroid gland ranging from the lack of thyroid gland (athyreosis) to a hypoplastic or ectopic gland. The remaining fraction (10–20%) of CH has been associated with hereditary defects in genes involved in thyroid hormone synthesis, termed as thyroid dysmorphogenesis (DH). Although TD is mainly considered as a sporadic disease, a genetic component has been

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proposed to be involved. In population studies, the prevalence of TD, as well as other thyroid abnormalities, has been significantly higher in first-degree relatives (6,7). Apart from a monogenic pattern of inheritance, multigenic and epigenetic mechanisms have been suggested to play a role in TD. To date, mutations in more than 15 genes have been reported to cause primary CH (8). All mutations affect either transcription factors that regulate thyroid-specific genes and thyroid development or genes responsible for thyroid hormone production. In addition to primary CH, mutations in genes regulating the hypothalamus–pituitary–thyroid axis can also lead to rare central hypothyroidism (1:50,000 newborns) (9).

This study established a targeted next-generation sequencing (NGS) panel for multigenic screening to identify the underlying genetic etiology of CH in hereditary and sporadic CH patients.

Materials and Methods

Study subjects, diagnostic criteria for CH, and laboratory measurements

Pediatric endocrinologists from different pediatric departments in Finland (Helsinki, Kuopio, Oulu, Turku, Vaasa, Seinäjoki) recruited patients with CH. The patients and their parents were asked to sign a written informed consent to take part in the study. The Ethics Committee of the Hospital District of Southwest Finland approved the study (108/180/2010).

The diagnosis of permanent CH was based on TSH and T4 screening test at birth and confirmation test results at two to three years of age after cessation of T4 treatment temporarily. CH screening in Finland is based on the measurement of TSH levels from umbilical serum at birth. Where an abnormal (TSH >40 mIU/L) result is found, serum T4 is determined from the same sample, and a confirmation sample (for both TSH and T4) is taken when the newborn is 72 hours old. This screening leads to an early start to therapy and has a low rate of false-positive cases (10). Iodine uptake or iodine accumulation tests were not performed routinely. Serum thyroglobulin (TG) levels at the time of recruitment were measured by commercial ELISA (Novatec, Baltimore, MD). DNA from whole-blood samples was isolated with a kit (Qiagen, Valencia, CA). A total of 34 DNA samples were sequenced using the targeted NGS panel containing the genes listed in Table 1. The cohort included both cases with thyroid dysgenesis and dysmaturagenesis. The other 17 DNA samples were obtained from sporadic cases, including 15 cases with permanent CH and two with hyperthyrotropinemia. Analyses included four CH patients with five known mutations in different genes responsible for CH (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/thy). Eleven CH samples were index cases from unrelated families with at least two affected family members. The summary of the clinical data is described in Figure 1 and in further detail in the supplementary section (Supplementary Materials and Methods).

Design and general performance of the sequencing panel for CH genes

The NGS panel consisted of 13 CH genes (Table 1). The aim was to cover all exons and exon–intron boundaries in genes known to cause CH using the Haloplex targeting system (Agilent, Santa Clara, CA). The custom primers were

TABLE 1. LIST AND THE COVERAGE OF THE GENES INCLUDED IN THE NGS PANEL USED FOR THE GENETIC CH SCREENING

Target ID	Regions	Coverage	High coverage (≥90%)	Low coverage (<90%)
<i>DUOX1</i> ^a	33	95.83%	31	2
<i>DUOX2</i>	32	95.82%	31	2
<i>IYD</i>	8	100.00%	8	0
<i>NKX2-5</i>	4	100.00%	4	0
<i>PAX8</i>	11	100.00%	11	0
<i>SLC26A4</i>	21	99.71%	21	0
<i>SLC5A5</i>	15	99.85%	15	0
<i>TG</i>	50	100.00%	50	0
<i>TPO</i>	18	100.00%	18	0
<i>TRH</i> ^a	2	100.00%	2	0
<i>TRHR</i> ^b	2	100.00%	2	0
<i>TSHB</i> ^b	2	100.00%	2	0
<i>TSHR</i>	11	100.00%	11	0

Three other known CH genes (*DUOXA2*, *FOXE1*, and *IGSF1*) were not included in the screening panel. Furthermore, the *NKX2-1* exons were previously sequenced.

^aNot previously linked to primary CH.

^bUsually associated with central CH.

NGS, next-generation sequencing; CH, congenital hypothyroidism.

designed using the Sure Design tool (Agilent Technologies, Santa Clara, CA) to generate 2219 amplicons with 250 bp length covering >99% of the target gene exon areas comprising 213 regions. The total amount of target bases was 38.157 kbp. Samples were sequenced using Illumina's MiSeq instrument (Illumina, San Diego, CA). After sequencing, 97% (208/213) of the targeted regions showed excessive coverage (>500), and three target region had coverage between 50 and 500. There were gaps in two targeted regions in the *DUOX2* (1/32 *DUOX2* targets) and *DUOX1* (1/33 targets) gene regions. All five mutations in the controls were identified with high read depth (>700) and expected allelic frequencies (Supplementary Table S1). Among all filtered variants confirmed by Sanger sequencing ($n=30$), no false-positive variants were observed.

Data analysis and filtering

An in-house developed analysis pipeline was used for the evaluation of raw fastq files generated by the MiSeq-sequencer. Cutadapt (<https://code.google.com/p/cutadapt/>) software served for adapter removal and read trimming. Reads shorter than 20 bp were abandoned. The remaining reads were mapped to the human reference genome hg19 using a BWA-MEM algorithm (<http://bio-bwa.sourceforge.net/>). Variant calling (SNVs and indels) was performed using four different variant caller tools: GATK HaplotypeCaller (www.broadinstitute.org/gatk/), SAMTools mpileup (<http://samtools.sourceforge.net/>), Atlas2 (<http://sourceforge.net/projects/atlas2/>), and Platypus (www.well.ox.ac.uk/platypus). All called variants were annotated using SnpEff (<http://snpeff.sourceforge.net/>), ANNOVAR (<http://annovar.openbioinformatics.org/>), and multiple different public databases (e.g., ExAC, 1000 Genomes, dbSNP, and ClinVar). Variants met the following inclusion criteria for the downstream analysis: located within the exon or splicing regions, a high or moderate effect on gene function, and an unknown or variant allele frequency <1% in the 1000 genomes variant database.

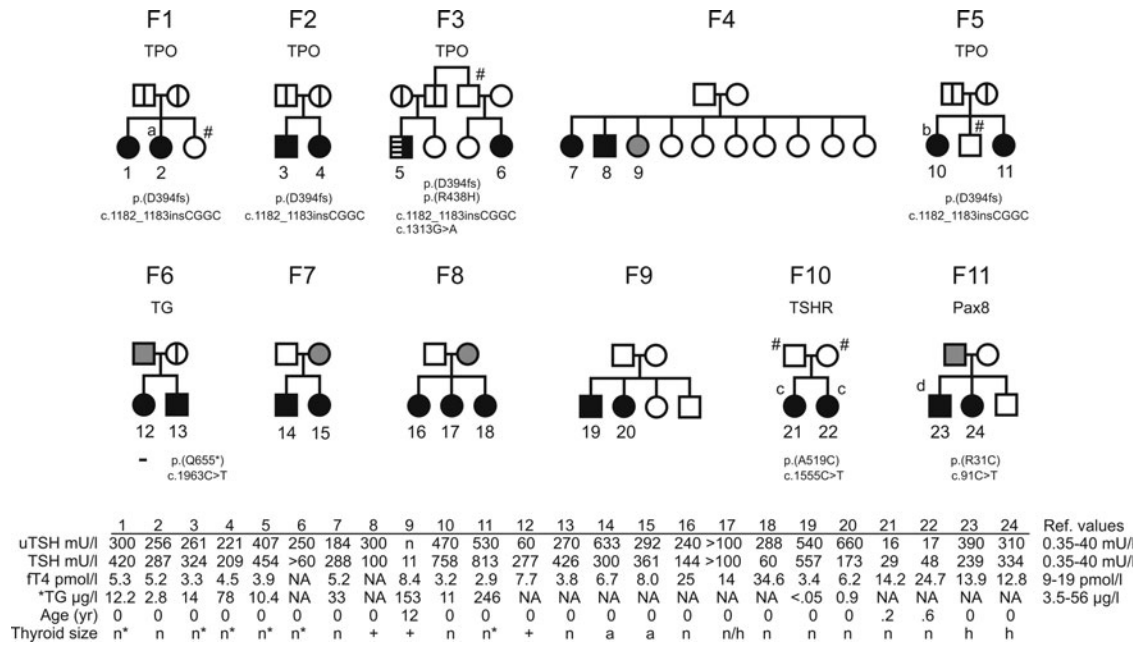


FIG. 1. Modified pedigrees and identified mutations of the familial congenital hypothyroidism (CH) cases with genetic and clinical data. The results of the thyroid function tests and thyroid size are aligned below. The reference values of umbilical blood thyrotropin (uTSH), and thyrotropin (TSH) and free thyroxine (fT4) serum hormone levels at three days of age or the time of diagnosis are included. Black, affected patients with CH; symbols with midline, asymptomatic heterozygous carriers; gray, adult onset hypothyroidism; white, no thyroid disease. Lined box (case 5) indicates the *TPO* R438H mutation. Serum thyroglobulin (TG) levels were measured at the time of enrollment into the study. The age (years) at time of diagnosis (0 = at birth) is shown. Thyroid size evaluated by thyroid ultrasound if data available (n, normal thyroid size and location; +, goiter; a, athyreosis/no thyroid gland not detected; h, hypoplastic or small thyroid; n*, no clinical signs of goiter during follow-up; #, no DNA available). The detailed clinical information of the families is described in the Supplementary Materials and Methods. NA, data not available. ^aPatient had mild hypotonia, delay of development (speech and motor development) and abnormal hearing response, ^babnormal hearing, ^cborn in week 29 + 3, or ^drenal agenesis.

The alignments at variant positions were visually inspected using the Integrative Genomics Viewer (www.broadinstitute.org/igv/; Supplementary Fig. S1). Variants that met these conditions and passed visual inspection were annotated using Condor (<http://bg.upf.edu/fannsdb/>). After the filtration procedure, the putative pathogenic variants were confirmed by Sanger sequencing using a commercial service (GATC Biotech, Germany). Furthermore, Sanger sequencing was used for the segregation analysis of the family members. Primers (sequences available upon request) were designed for all variants listed in Tables 2 and 4, used for genomic polymerase chain reaction to amplify DNA flanking the variant. All three

exons of *NKX2.1* were sequenced as previously described (11). Furthermore, array-based comparative genomic hybridization (CGH) and multiplex ligation-dependent gene (MLPA) analysis for *NKX2.1* gene deletion and duplication was performed at CeGAT laboratories (GeCAT GmbH, Tübingen, Germany) for a sporadic case with nearly lethal respiratory failure at birth.

Protein modeling and in vitro experiments

The impact of *TPO* and *PAX8* mutations on protein function was investigated using protein modeling, as described in

TABLE 2. PATHOGENIC MUTATIONS IDENTIFIED FROM FAMILIAL CH CASES

Fig. 1 no.	Gene	GeneBank	Base change	Protein change	ExAC MAF	Mono/biallelic	Read depth	Classification
2	<i>TPO</i>	000547.5	c.1182_1183insCGGC	D394fs	—	Biallelic	11	A
3	<i>TPO</i>	000547.5	c.1182_1183insCGGC	D394fs	—	Biallelic	3	A
5	<i>TPO</i>	000547.5	c.1182_1183insCGGC	D394fs	—	Monoallelic	4	A
5	<i>TPO</i>	000547.5	c.1313G>A	R438H	—	Monoallelic	997	B
10	<i>TPO</i>	000547.5	c.1182_1183insCGGC	D394fs	—	Biallelic	8	A
13	<i>TG</i>	003235.4	c.1963C>T	Q655*	0.0001	Monoallelic	887	B
21	<i>TSHR</i>	000369.2	c.1555C>T	R519C	0.00004	Monoallelic	997	A
23	<i>PAX8</i>	003466.3	c.91C>T	R31C	—	Monoallelic	992	A

Novel *TG* and *TPO* mutations shown in bold.

A, pathogenic mutation (based on segregation, literature, and *in vitro* experiments); B, likely pathogenic mutation (predicted pathogenicity *in silico* and/or modeling analysis); ExAC MAF, allelic frequency in The Exome Aggregation Consortium database.

more detail in the Supplementary Materials and Methods. A luciferase reporter assay was performed to evaluate the pathogenicity of the mutations in *NKX2.1* and *PAX8*, as described in the Supplementary Materials and Methods. The wild-type pcDNA3.1-NKX2.1 plasmid containing the short version of NKX2.1 was obtained from Addgene (plasmid 49989; Addgene, Cambridge, MA) (12). The wild-type pcDNA3-PAX8 plasmid was a kind gift from Dr. Knut Krohn (University of Leipzig, Leipzig, Germany).

Results

Results from the familial cases

Among the 11 index cases with familial CH, the following thyroid or associated phenotypes were found: goiter, athyrosis, thyroid gland hypoplasia, kidney agenesis, and, in one family, the cases were twins born prematurely at gestational week 29+3 (Fig. 1). Other families showed no obvious phenotypic signs or syndromes. Among 11 families, five were from the same district in Finland. In four of those families, a frameshift (fs) mutation (c.1182_1183ins CGGC) in the *TPO* gene in exon 8 was identified. This fs mutation altered the amino acid sequence of TPO after amino acid 394, and introduced a premature stop codon in exon 9. This change led to a large truncation of TPO and its peroxidase domain with no expected activity (Fig. 2). In three out of four families, this *TPO* mutation was homozygous in affected children. The thyroid ultrasound was normal, and no clinical goiter was observed in the affected cases during the clinical follow-up. Parents were heterozygous carriers, and all had a normal thyroid size and function. In family F3 (Fig. 1, F3), two cousins (#5 and #6) with CH had a heterozygous *TPO*fs mutation. Besides this mutation, one of them (#5) also had a rare novel heterozygous c.1313G>A, p.R438H mutation, which is absent in databanks and located in a well-established functional TPO domain. Based on the structural protein modeling, the histidine mutant at position 438 impairs the important intramolecular interaction. This led to the modification of the structural adjustment inside the domain, which is essential for regular enzymatic function of TPO (Fig. 2). The heterozygous *TPO*fs mutation was present in the mother and the R438H mutation in the father with normal thyroid function tests (TFTs). Thus, these compound heterozygous mutations of *TPO* most likely explain the development of CH in this patient. However, only the *TPO*fs mutation could be identified, not another *TPO* variant from his cousin who also suffered from CH.

In family F10, a heterozygous mutation in the *TSHR* 1555C>T, p.R519C was found in identical twins, born prematurely at week 29+3 (Fig. 1 and Supplementary Materials and Methods). In the initial CH screening, umbilical TSH serum levels were under the cutoff level (16 and 17 mIU/L; reference value >20 mIU/L), but serum TSH levels were elevated during follow-up. Serum TSH concentrations were markedly elevated, but free thyroxine (fT4) concentrations were within the reference range at the time of diagnosis (at two and six months of age), indicating compensated hypothyroidism. During the follow-up, both cases needed an increasing dose of T4 supplementation to maintain TSH at normal levels. Despite the adequate T4 replacement, one of the twins had a mild developmental delay, probably due to the premature birth. In the T4 withdrawal test at three years of age, serum TSH levels rose markedly over the reference value, and

fT4 levels decreased but remained in the reference range in both cases. Their mother had a goiter and fluctuations in TFTs, but did not require any T4 medication. Additionally, two *TSHR* variants, Pro52Thr and Asp36His, were identified among the study subjects, but they were predicted to reflect polymorphisms without functional consequences (13).

Family F11 (Fig. 1) included two children with CH. Both affected individuals were found to carry a heterozygous *PAX8* c.91C>T, p.R31C mutation causing an amino acid change in the PAX8 protein (Table 2). The detected arginine to cysteine mutation at position 31 leads to a loss of structural-functional constraints and intermolecular contacts to the DNA response element and thus a loss of functionality (Fig. 3). Both cases showed serum TSH levels >200 mIU/L, fT4 was in the low range of the reference values, and they had a small or hypoplastic thyroid gland evaluated by ultrasound. In addition, one of the affected patients had a right kidney agenesis. The father was diagnosed with nonautoimmune hypothyroidism at 30 years of age, and sequencing of *PAX8* revealed that he was also a carrier of the *PAX8* c.91C>T mutation. The mother and other siblings had normal TFTs and no alteration in the *PAX8* sequence. In line with the modeling, the *in vitro* tests demonstrated that the transcriptional activity of the *PAX8* mutant was almost completely lost compared with the WT protein (Fig. 3).

The details of the mutations among the familial cases are described in Table 2. Four families (Fig. 1) with hereditary CH had no significant mutations in the target genes. However, in one family (F6) with goitrous CH, the index case had a rare novel heterozygous *TG* mutation. It introduced a premature stop of translation at position 655 of the TG protein and is predicted to be pathogenic based on the *in silico* analysis. However, it was not segregating with the phenotype, but present in the healthy father, and not in the other affected sibling.

Results from the sporadic CH cohort

The sporadic cohort included CH patients with a normal thyroid, hypoplasia, or agenesis of the thyroid. Four patients suffered from respiratory distress syndrome (RDS) or mild respiratory problems at birth. Furthermore, three cases were diagnosed with minor heart malformations, including ventricular septal defect (VSD), persistent ductus arteriosus (PDA), and persistent foramen ovale (PFO). Otherwise, the patients did not have other significant diagnoses, and they did not suffer from developmental problems (Table 3).

Among these cases, one patient with a typical brain-lung-thyroid syndrome had nearly lethal RDS and pulmonary dysfunction at birth (Tables 3 and 4, #73). In the sequencing analysis, the *NKX2.1* sequence was normal. Due to the typical phenotype, the presence of haploinsufficiency was tested with multiplex ligation-dependent gene analysis (MLPA), which revealed a large hemiallelic deletion covering all three exons. In further analysis, a larger 1.1 Mb microdeletion was detected in location 14q13q21.1. This *de novo* deletion included six protein-coding genes (*SFTA3*, *NKX2.1*, *NKX2.8*, *PAX9*, *SLC25A21*, and *MIPOLI1*). Despite adequate and prompt T4 replacement, the patient developed severe mental and motor retardation, and required continuous medication for interstitial lung disease. The parents had no thyroid or genetic abnormalities. In addition to this large hemiallelic

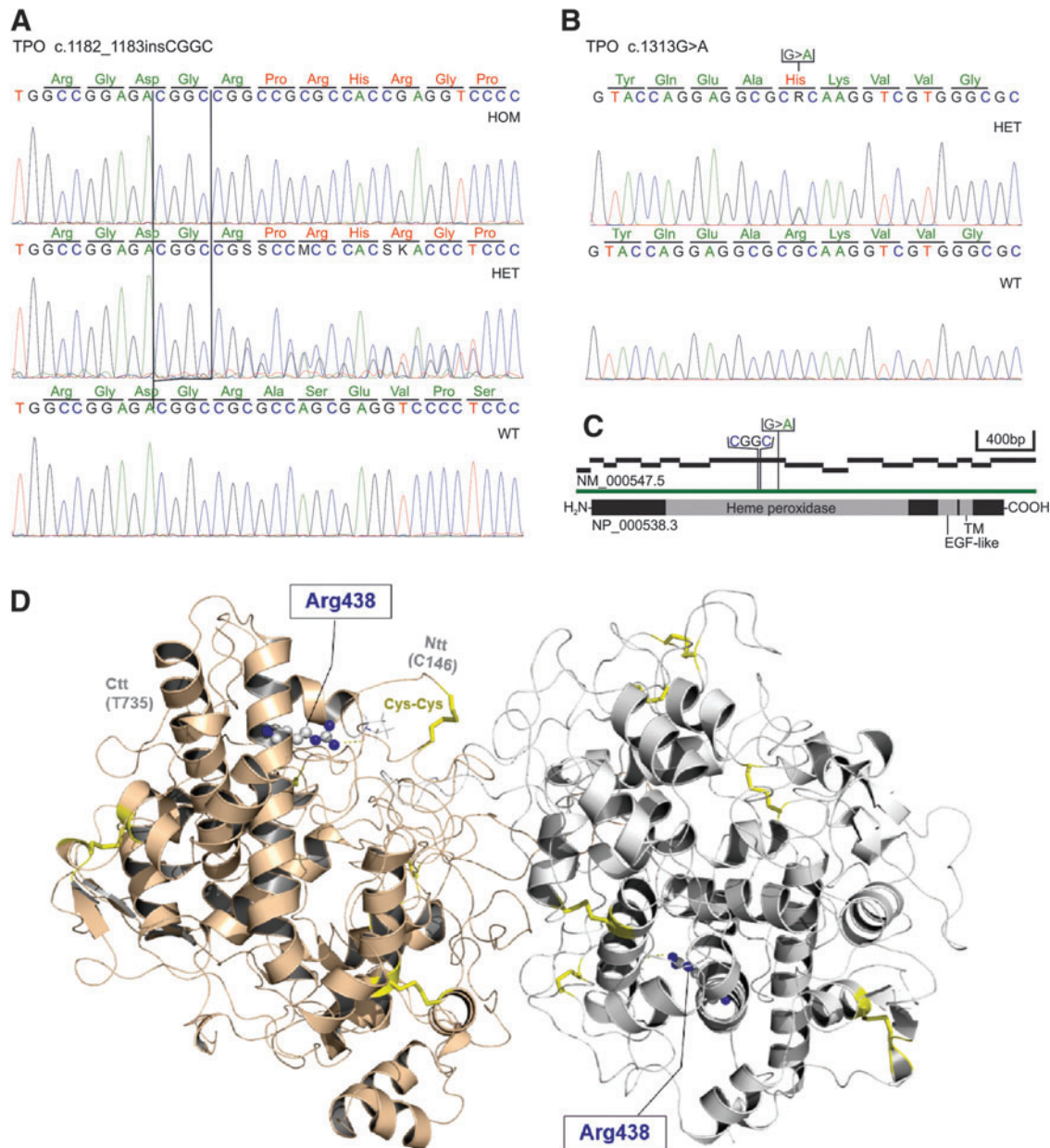


FIG. 2. Confirmation of the *TPO* mutations identified from familial cases by Sanger sequencing and protein modeling. Sanger chromatograms of (A) the heterozygous (HET) and homozygous (HOM) *TPO* c.1182_1183insCGGC mutant and (B) *TPO* c.1313G>A point mutation compared with WT sequence. (C) A schematic picture of *TPO* with the localization of the mutations. (D) Dimeric model of the myeloperoxidase-like domain of *TPO* based on a homologous dimeric structure of myeloperoxidase. The homology model contains residues from Cys146-Thr735, whereby only the backbone is visualized (ribbon-tube cartoon). The *TPO* protomers are colored differently (the heme group, ions, and glycosylations are not represented). Cysteine disulfide-bridges (yellow sticks) are involved in maintaining the quaternary structure. TM, transmembrane domain. The side-chain of arginine at position 438 is connected via an H-bond to the loop-backbone at proline 153 and functions in consequence as a structural constraint between both domain fragments. The histidine mutant at position 438 likely fails establishing this important intramolecular interaction and leads to modification of the structural adjustment inside the domain.

NKX2.1 deletion, two additional novel *NKX2.1* variants were detected. One sporadic CH patient (Tables 3 and 4, #71) had a small 9 bp deletion, c.793_801del, in *NKX2.1*. The patient was born premature at week 36 and required respiratory support at the newborn intensive care unit for two days, but no additional lung or neurological phenotype became apparent after the newborn period. The 9 bp deletion c.793_801del, p.G264-

266del leads to the removal of three glycine residues from the glycine-rich area of the protein. However, the deletion had no impact on the transactivation of the *TG* promoter, as shown by a luciferase reporter assay (Supplementary Fig. S2). Moreover, the deletion was present in the father with normal TFTs and no sign of thyroid autoimmunity. Furthermore, a patient with elevated TSH levels, fT4 levels at reference range, and thyroid

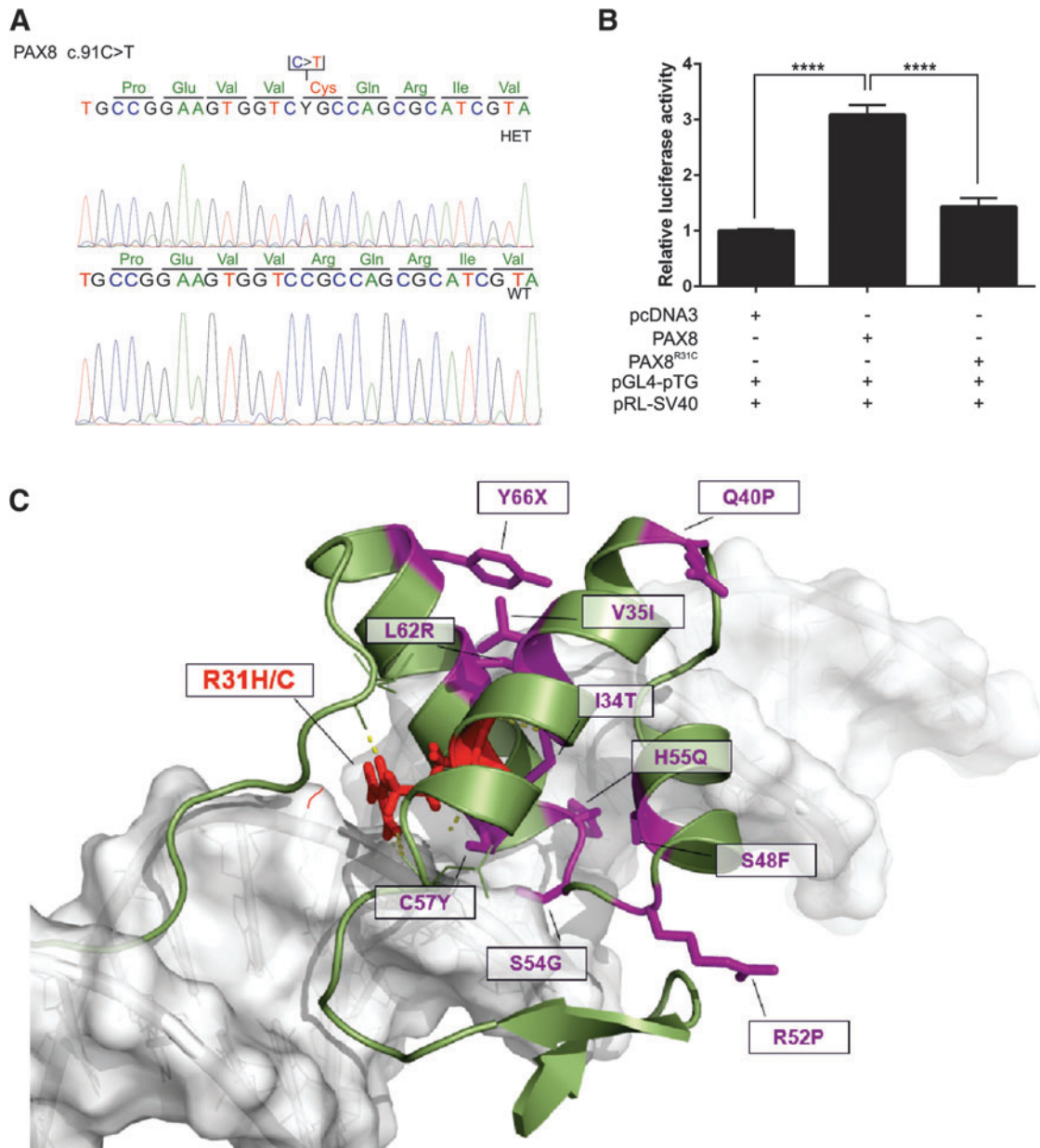


FIG. 3. Characterization of the *PAX8* R31C mutation in a familial CH case. **(A)** Sanger chromatogram visualizing the mutated allele in this family. **(B)** A loss of transactivation activity of the *PAX8* R31C mutant compared with wild type using a *TG* promoter luciferase reporter assay. Bars represent means \pm standard error of the mean from experiments performed on three separate days ($n = 15$; **** $p \leq 0.0001$). **(C)** Three-dimensional *PAX8* model with highlighted arginine at position 31. The crystallized *PAX5* structure (backbone presentation green) together with a DNA response element (white backbone and translucent surface) was used to model *PAX8* protein fragments. Examples of other known pathogenic *PAX8* mutations are labeled with magenta sticks. Mutations at the hydrophobic inner core disturb the tight package between the helices (such as Ile34Thr or Cys57Tyr). An intermolecular H-bond from Arg31 contacts the DNA and mediates the proper justification of the protein and the DNA toward each other.

hemiagenesis was included in the genetic analysis (Tables 3 and 4, #40). Interestingly, he was carrying a c.313G>A missense mutation (p.V105M) in *NKX2.1*, but the mutation was present also in the healthy parent, and it did not affect the transactivation of the *TG* promoter, indicating this mutation is most likely benign (Supplementary Fig. S2).

Rather unexpectedly, several rare *TG* mutations were observed in our sporadic CH cohort. In one case with permanent and apparently sporadic CH (Tables 3 and 4, #77), compound

heterozygous mutations were identified in *TG*. Both parents carried one of the mutations and had normal TFTs. The c.1963 C>T mutation, p.Q655*, also observed in a familial case (Fig. 1, F6, #13), caused a premature stop of translation at position 655 of the *TG* protein. The other *TG* mutation c.4378G>A, p.V1460I is also rare (MAF <0.0003) and predicted to be highly pathogenic in standard protein function prediction programs. The serum *TG* levels in this patient were undetectable, and no goiter was seen in a computed tomography scan of the neck at birth or

TABLE 3. CLINICAL CHARACTERISTICS OF THE SPORADIC COHORT

No.	<i>uTSH/uT4/ft4</i>	<i>TSH</i>	<i>ft4</i>	<i>TG</i>	<i>Dysfunction, morphology</i>	<i>Age (days), sex</i>	<i>Other diagnosis, affected family members</i>
42	240/—	270 (3 days)	10.3 (3 days)	—	CH, —	0, F	RDS
54	373/—	>100 (3 days)	6.0 (3 days)	—	CH, —	0, M	—
55	880/4/—	>100 (3 days)	<3.0 (3 days)	—	CH, —	0, M	Hyperbilirubinemia
56	530/—	550 (4 days)	8.2 (4 days)	1.86	CH, —	0, F	—
57	44/108/13.8	23 (3 days)	25.1 (3 days)	9.66	CH, —	0, M	Transient tachypnea, hypoglycemia
59	83/—	64 (3 days)	20.4 (3 days)	4.8	CH, —	0, F	VSD, M has abnormal TFTs and anomalous thyroid
70	111/—	—	—	—	CH, hypoplasia	0, M	—
71	520/—	258 (3 days)	14.4 (3 days)	—	CH, agenesis	0, F	Prematurity (36+0)
73	400/—	170 (3 days)	<5.2 (3 days)	—	CH, —	0, M	RDS, PH, interstitial lung disease, mental and retardation
77	84/—	78 (3 days)	19.5 (3d)	<0.05	CH, normal	0, F	PDA, PFO
78	88/113/10.8	53 (3 days)	15.8 (3d)	0.89	CH, —	0, M	—
90	250/83/—	375 (8 days)	—	—	CH, agenesis	0, F	VSD, M thyroid Ca
92	12/—	13 (13 days)	14.8 (13d)	—	CH, hypoplasia	27, M	Gemini
99	315/67/7.8	380.9 (2 days)	7.5 (2d)	2.19	CH, normal	0, F	Asthma
103	670/116/12	250 (3 days)	16.3 (3d)	4.99	CH, —	0, M	—
39	9/—	9.1	16.1	—	hyperTSH, —	5 months, F	Mild developmental delay
40	16/—	8.3 (5 years)	15.4 (5y)	—	hyperTSH, hemigenesis	5 years, M	—, M has AIH

Reference values: *uTSH/uT4/ft4* (<40 mIU/L, >120 nM/L, >10 pmol/L); control *TSH* and *ft4* at 3 days old 0.4–6 mIU/L and 9–19 pmol/L, serum *TG* levels 3.5–56 µg/L.

uTSH/uT4/ft4 = hormone levels measured at birth from umbilical cord; *TSH* and *ft4* = confirmation of the positive screening values (age in parentheses).

uTSH, umbilical thyrotropin; *uT4*, umbilical thyroxine; *ft4*, free thyroxine; *TG*, thyroglobulin; RDS, respiratory distress syndrome; VSD, ventricular septal defect; TFTs, thyroid function tests; PH, pulmonary hypertension; PDA, persistent ductus arteriosus; PFO, persistent foramen ovale; M, mother; Ca, carcinoma; AIH, autoimmune thyroid disease.

TABLE 4. DETAILED INFORMATION AND CLASSIFICATION OF VARIANTS IDENTIFIED FROM SPORADIC CH CASES

No.	<i>Gene</i>	<i>GeneBank</i>	<i>Base change</i>	<i>Protein change</i>	<i>ExAC MAF</i>	<i>Read depth</i>	<i>Classification</i>
42	<i>SLC26A4</i>	NM_000441.1	1796C>T	A599V	0.00019	915	B
42	<i>NKX2.5</i>	NM_001166176.1	c.428G>A	R143Q	0.00082	994	C
55	<i>SLC26A4</i>	NM_000441.1	c.1363A>T	I455F	—	640	C
56	<i>DUOX1</i>	NM_017434	c.656C>T	P219L	0.00010	989	C
57	<i>TG</i>	NM_003235.4	c.353C>T	P118L	0.01471	510	C
57	<i>DUOX2</i>	NM_014080.4	c.908C>G	P303R	0.01067	564	B
59	<i>SLC26A4</i>	NM_000441.1	c.2326C>T	R776C	0.00200	496	C
71	<i>NKX2.1</i>	NM_001079668	c.793_801del	G264-266del	—	—	C [#]
73	<i>NKX2.1</i>	NM_001079668	hemidel	—	—	—	A
77	<i>TG</i>	NM_003235.4	c.1963C>T	Q655*	0.00013	329	B
77	<i>TG</i>	NM_003235.4	c.4378G>A	V1460I	0.00020	182	B
90	<i>DUOX2</i>	NM_014080.4	c.518A>G	A173S	—	997	C
99	<i>TG</i>	NM_003235.4	c.6130C>T	R2044C	0.00005	996	B
99	<i>TG</i>	NM_003235.4	c.5921T>C	M1974T	0.08417	999	C [#]
103	<i>SLC26A4</i>	NM_000441.1	c.1790T>C	L597S	0.00826	988	B
103	<i>TG</i>	NM_003235.4	c.3416C>T	S1139L	0.00030	719	B
39	<i>TRH</i>	NM_007117.4	c.212C>T	A71V	0.00039	997	C [#]
39	<i>TSHR</i>	NM_000369.2	c.106G>C	D36H	0.00602	966	C [#]
40	<i>NKX2.1</i>	NM_001079668	c.313G>A	V105M	—	—	C [#]

Novel mutations in known CH genes shown in bold.

A, pathogenic mutation (based on segregation, literature, and *in vitro* experiments); B, likely pathogenic mutation (predicted pathogenicity in silico analysis); C, functional effect unclear; C[#], likely benign mutation (based on *in vitro* test and/or segregation); *, stop codon.

observed during follow-up. The other case with nongoitrous CH (Tables 3 and 4, #99) had a low frequency novel heterozygous c.6130C>T, p.R2044C *TG* mutation (present in 1/6614 alleles in the Finnish population), which is predicted to be pathogenic *in silico*. The adjustment of the filtering parameters of the NGS analysis and search for compound heterozygous variants in the *TG* gene revealed another c.5921T>C, p. M1974T missense variant in this case. However, there are several cases with homozygosity for this allele in the ExAC database. The variant, although present in only 6% of the Finnish population is present in fairly high frequency in certain populations, indicating that it is probably a polymorphism. The healthy parents were carriers of one of the mutations. Additionally, a combination of rare heterozygous *TG*, p.S1139L, and *SLC26A4*, p.L597S mutations was present in a sporadic case with normal thyroid gland (Tables 3 and 4, #103). The extended family history revealed that he had two other CH cases in the distant family, but DNA could not be obtained from these patients.

A few rare and likely pathogenic heterozygous *DUOX2* (p.P303R and novel p.A173S) and *SLC26A4* (p.I455F, p.R776C, p.L597S, and a novel p.A599V) variants were present among the study subjects (Table 4). A search for more common compound heterozygous variants was then performed, but the segregation or pathogenicity of these mutants could not be confirmed. Sanger sequencing of the *DUOX2* exons from patients with *DUOX2* mutations did not reveal any novel mutations either.

Discussion

To date, more than 15 causative genes have been linked to CH. The large number of genes poses a challenge to select target genes for Sanger sequencing. The initially subtle clinical phenotype and the lack of detailed diagnostic tests, which are often not performed routinely, may hamper the selection of possible target genes.

The present study established a high-throughput NGS panel to enable cost-effective, efficient, and multigenic screening covering nearly all known CH target genes. To the best of the authors' knowledge, such a broad screening has not been reported in CH previously. Using this panel, molecular causes for CH were identified in 6/11 families with different forms of familial CH. Furthermore, several pathogenic and likely pathogenic mutations were found among sporadic cases. Due to the high coverage of the NGS screen, it is unlikely to have missed any mutations among the exons of the candidate genes in the remaining five families with negative findings. Hence, these families may perhaps carry intronic mutations, larger deletions in the respective gene areas, or novel CH gene mutations, which are not detected by the current panel. As demonstrated in this study by the patient with typical brain–lung–thyroid syndrome, and also shown in other studies (11), a common pitfall of the NGS and conventional sequencing is that larger deletion(s) will go unnoticed. Hence, recognizing these deletions requires other methods such as multiplex ligation-dependent gene analysis or comparative genomic hybridization techniques, which should be considered in specific cases. Identification of the deletions or insertions in NGS data analysis is challenging, and the common algorithms for recognizing them yields variable results and are not yet in standard use (14).

In the familial cases, pathogenic mutations were found in the *TPO*, *PAX8*, and *TSHR* genes. Interestingly, the *TPO* frameshift mutation leading to an early truncation of the protein was present in four of these families. When present in a homozygous form, this mutation has been shown to inactivate the *TPO* function completely, which results in elevated TSH, low serum T4 levels, and nearly total release of iodine in the perchlorate test (15). Similar mutations in this region have been described and shown to be the most prevalent cause for thyroid dysmorphogenesis in several populations (15–17). Although the *TPO* mutations are typically associated with thyroid goiter, no goiter was observed among of our homozygous *TPO* c.1182_1183insCGGC patients or in their heterozygous parents. Thus, other factors such as dietary iodine supply, efficacy of the thyroxine replacement, or other genetic findings may be involved in this phenotype. In two affected cousins with heterozygous *TPO*fs mutations, one carried another novel *TPO* missense mutation, *R438H*, which most likely explains the phenotype. The insights from *TPO* homology models suggested that the *R438H* mutation likely impairs the enzymatic function by modification of the intramolecular structure (Fig. 2D). The secondary and quaternary structure of the enzyme might be modified due to interruption of essential H-bonds important for structural adjustments. However, in the other case, no additional *TPO* mutations could be detected. This may be due to another pathogenic mutation located in the promoter or intron areas, which were not covered by the panel, or, for example, due to monoallelic expression of the mutant gene, as suggested by Fuggazola *et al.* (18).

NKX2.1 is a transcription factor playing an important role in thyroid development. Additionally, it has a crucial impact on brain and lung development. Typically, the patients with impaired *NKX2.1* function have both severe pulmonary and neurological symptoms (11,19). None of the familial cases had any typical brain–lung–thyroid phenotype. In line with this, no alteration in *NKX2.1* was found among the hereditary cases. In contrast, a sporadic case with severe pulmonary dysfunction and neurological symptoms was found to carry a large hemiallelic deletion in the *NKX2.1* area in further analysis, although the *NKX2.1* sequence appeared normal in the initial screening. This highlights the importance of using alternative methods to exon sequencing alone in the genetic analysis of patients suspected with this CH phenotype. Interestingly, two novel alterations were detected in *NKX2.1* in the cohort. One case showed mild respiratory adaptation problems at birth without any further lung phenotype, and the other case had non-autoimmune hypothyroidism and hemiagenesis, but no additional phenotypic features. However, the *in vitro* *TG* transactivation tests indicate that the c.313G>A missense and the c.793_801del deletion mutants do not have any effect on the transactivation of the *TG* promoter compared to wild-type *NKX2.1*. Moreover, the presence of the variants in healthy parents suggested that these were most likely benign. However, only the transactivating activity of these mutants was tested, and therefore the possibility that the *NKX2.1* mutations could affect *NKX2.1* protein interactions, for example, cannot be excluded.

The heterozygous *PAX8* mutation R31H was one of the original *PAX8* mutations described and shown to lack DNA binding activity *in vitro* (20). Here, a family with another heterozygous mutation, R31C, at this position was identified. The two siblings have CH and a hypoplastic thyroid, and one of them also has a unilateral kidney agenesis. Based on the

protein modeling (Fig. 3) and transactivation assay, this *PAX8* R31C mutation seems to inactivate the transcription factor activity completely, as reported previously for the R31H mutation (21–23), suggesting that this codon is a mutational hot spot.

Loss-of-function mutations in the *TSHR* have been reported as a common cause of CH in several populations (24). Contrary to gain-of-function mutations, which are always heterozygous, the occurrence of inactivating *TSHR* mutations can be heterozygous, compound heterozygous, or homozygous (25,26). In the present cohort, twins were identified harboring a R519C *TSHR* mutation in transmembrane helix (TMH) 3. Position R519 is highly conserved within family A of G-protein coupled receptors (27). In line with this, the functional characterization of *TSHR* R519C and R519G mutants in the study by Tsunekawa *et al.* demonstrated reduced ligand binding and impaired activation of G_{sz} and $G_{q/11}$ compared with the wild-type receptor (28). In addition, several studies suggested that heterozygous inactivating mutations may be associated with CH and that the mutant *TSHR* could have a dominant negative effect on the activity of the wild-type receptor (29). This occurs mainly via intracellular entrapment of the *TSHR* dimer/oligomer between wild-type and mutated receptors, which are formed shortly after translation.

Mutations in *TG* are a common cause of CH, often resulting in the development of a large goiter in the affected cases. Unexpectedly, among the 11 pedigrees with familial CH, no disease causing *TG* mutations was found. In one family with goitrous CH, a heterozygous *TG* variant was identified in two children, but it was not segregating with the phenotype in the family, nor was any other variant of *TG* detected. Surprisingly, a few likely pathogenic monoallelic *TG* mutations were found in apparently sporadic CH patients. In two cases, the CH was most probably caused by rare compound heterozygous mutations in *TG*.

Two rare *DUOX2* mutations were found in sporadic cases. The *DUOX2* p.P303R mutation has previously been shown to have a slightly lower activity compared to the wild type (30). However, no other mutation could be identified in *DUOX2* or in *DUOXA2* in this patient. The *DUOX2* p.A173S variant has not been reported previously to the best of the authors' knowledge. These mutations were monoallelic, and we could not obtain samples from relatives to confirm segregation with the phenotype, which makes it challenging to draw conclusions regarding these variants.

The rare *SLC26A4* variants that were detected (p.A599V, p.I455F, p.R776C, and p.L597S) from the CH cases have partly been characterized before. Both the p.L597S and the p.R776C mutants have been detected in Pendred syndrome cases, but the functionality seems to be indistinguishable from the wild-type protein, and the pathogenicity of these mutations is therefore unclear (31,32). The p.I455F mutant has been found in a heterozygous state in controls with normal hearing (33). To the authors' knowledge, the p.A599V variant has not been detected before in cases with CH or Pendred syndrome.

In summary, the targeted NGS panel of nearly all currently known CH genes provides an efficient, cost-effective, and multigenic screening tool for gene mutations in the coding region of these genes. It may be an additional tool for clinicians to identify the genetic etiology accurately of the selected CH patients in the future, especially in dysmorphic, familial, and syndromic CH cases. Moreover, it may serve as a valuable prescreening method prior to exome or genome analyses.

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Author Disclosure Statement

The authors declare no conflicts of interest.

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