

RESEARCH ARTICLE

High frequency of mutations in 'dysmorphogenesis genes' in severe congenital hypothyroidism

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Abstract

Objective

Results of the screening of disease causative mutations in congenital hypothyroidism (CH) vary significantly, depending on the sequence strategy, patients' inclusion criteria and bioinformatics. The objective was to study the molecular basis of severe congenital hypothyroidism, using the next generation sequencing (NGS) and the recent guidelines for assessment of sequence variants.

Design

243 patients with CH (TSH levels at neonatal screening or retesting greater than 90 mU/l) and 56 control subjects were included in the study.

Methods

A custom NGS panel targeting 12 CH causative genes was used for sequencing. The sequence variants were rated according to American College of Medical Genetics and Genomics (ACMG) guidelines.

Results

In total, 48 pathogenic, 7 likely pathogenic and 57 variants of uncertain significance were identified in 92/243 patients (37.9%), while 4 variants of uncertain significance were found in 4/56 control subjects (7.1%). 13.1% (12/92) of the cases showed variants in 'thyroid dysgenesis' (TD) genes: *TSHR*, n = 6; *NKX2-1*, n = 2; *NKX2-5*, n = 1; *PAX8*, n = 3. The variants in 'dysmorphogenesis' (DH) genes were found in 84.8% (78/92) of cases: *TPO*, n = 30; *DUOX2*, n = 24; *TG*, n = 8; *SLC5A5*, n = 3; *SLC26A4*, n = 6; *IYD*, n = 1. 8 patients showed oligogenic variants. The majority of variants identified in DH genes were monoallelic.

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Conclusions

In contrast to earlier studies demonstrating the predominance of TD in severe CH, the majority of variants identified in our study were in DH genes. A large proportion of monoallelic variants detected among DH genes suggests that non-mendelian mechanisms may play a role in the development of CH.

Introduction

Congenital hypothyroidism (CH) is a partial or complete loss of function of the thyroid gland that affects infants from birth, being the most common inborn endocrine disorders, with a prevalence of 1 in 3000–4000 newborns [1]. Historically, insights into the etiology of CH were given by the results of scintigraphy and ultrasonography studies, according to which, thyroid dysgenesis (TD) was defined in 80–85% of patients, while the remaining 15–20% of cases were believed to be due to thyroid dyshormonogenesis (DH) [2,3]. At least 12 genes have been described that are involved in the pathogenesis of CH, part of which were shown to be involved in thyroid dysgenesis (TD) [4] (*TSHR* [5], *PAX8* [6], *NKX2-5* [7], *FOXE1* [8], *NKX2-1* [9,10]), while the others were linked to the defects in biosynthesis of thyroid hormones, i.e. dyshormonogenesis (DH) (*TPO* [11], *IYD* [12], *SLC26A4* [13], *TG* [14], *SLC5A5* [15], *DUOX2* [16], *DOUXA2* [17]) [18]. Studies on the molecular basis of CH in the pre-NGS era were usually performed in patients with specific clinical or thyroid imaging characteristics and were focused on a limited number of genes and (or) a small number of cases. Such studies revealed molecular origin of CH in less than 10% of cases [19–22]. The introduction of the next generation sequencing (NGS) made the studies in CH more efficient and showed a higher rate of mutations in subjects with CH [23–28].

In the current paper we present results of NGS in 243 Russian patients with CH. In this study covering the largest patients' cohort reported to date we have included cases only with severe CH (TSH at diagnosis > 90 mU/L). Assessment of pathogenicity of sequence variants was based on the American College of Medical Genetics and Genomics (ACMG) guidelines [29], which eliminated from analysis single nucleotide variants with minor allele frequency (MAF) greater than 0.001.

Subjects and methods

Subjects

This study was approved by the local ethics committee of the Endocrinology Research Centre (Protocol №12 dated 22.10.2014). Informed written consents were obtained from the patients or (and) the parents.

243 patients (94 males, 149 females) with severe CH, defined as TSH levels at neonatal screening or re-testing greater than 90 mU/L, were included in the study. At the time of the study the age of the patients ranged from 4 weeks to 18 years (median, 4.5 years).

56 subjects (24 males, 32 females) were included in the control group. The inclusion criteria were normal levels of TSH and free T4, no thyroid antibodies, no changes according to the thyroid ultrasound.

DNA sequencing

Genomic DNA was extracted from peripheral leukocytes using PureLink® Genomic DNA Mini Kits (Thermo Scientific, USA). A custom Ion Ampliseq™ panel (Ion Torrent, Thermo

Scientific, USA) targeting 12 genes associated with hypothyroidism (*TPO*, *PAX8*, *NKX2-5*, *IYD*, *SLC26A4*, *TG*, *FOXE1*, *NKX2-1*, *DUOX2*, *DOUXA2*, *TSHR*, *SLC5A5*) was used for DNA library preparation. Sequencing was performed using Personal Genome Machine (PGM) semiconductor sequencer (Ion Torrent, Thermo Scientific, USA). Bioinformatics analysis was carried out using Torrent Suite 4.2.1 (Thermo Scientific, USA) and ANNOVAR ver. 2014 Nov12 software packages [30]. The results of the NGS were confirmed by Sanger sequencing using Genetic Analyzer 3130 sequencer (Life Technologies, USA). Interpretation of the sequencing results and assessment of the pathogenicity of sequence variants were performed according to the ACMG guidelines [29]. Sequence variants rated as 'benign' or 'likely benign' were excluded from the analysis. A description of the sequence variants was carried out in accordance with the recommendations of den Dunnen and Antonarakis [31].

MLPA

A multiplex ligation-dependent probe amplification (MLPA) was carried out on 24 patients: one patient with suspected deletion of multiple exons in *PAX8* gene, as determined by the NGS coverage analysis; and 23 patients with a single heterozygous mutation in *TPO* or *TSHR* genes. SALSA MLPA probemix P319 set (genes *TPO*, *PAX8*, *FOXE1*, *NKX2-1* and *TSHR*, MRC-Holland, Netherlands) and a standard set of reagents SALSA MLPA EK1-FAM (MRC-Holland, Netherlands) were used. Data processing was carried out using software [Coffalyser.Net](#) (MRC-Holland, Netherlands).

Statistical analysis

Pearson χ^2 and odds ratio were applied to analyze the results of the study.

Results

NGS identified 63 different sequence variants in 92 of 243 patients (37.9%). Homozygous variants were identified in 12.0% (11/92), compound heterozygous variants in 13.0% (12/92), heterozygous variants in 66.3% (61/92), 8.7% variants were identified in two genes (8/92). 84.8% (78/92) variants were in the DH genes (*TPO*, *IYD*, *SLC26A4*, *TG*, *SLC5A5*, *DUOX2*, *DOUXA2*), 13.1% (12/92) of the variants were identified in the TD genes (Fig 1). Variants in two groups of genes were identified in 2 patients (2.1%, 2/92).

In our study the majority of variants were found in *TPO* gene (in 30 of 92 patients, 32.6%) (Table 1). Defects in *TPO* gene included insertions and deletions with frameshift ($n = 4$), nonsense variants ($n = 1$), missense variants ($n = 13$), splice-site variants ($n = 2$). No deletions or insertions were identified in patients from this group using MLPA. The second most frequent findings were changes in *DUOX2* gene (26.1%, 24/92), we identified a deletion with frameshift, 2 nonsense and 7 missense variants (Table 2). 8 patients (8.7%) showed variants in *TG* gene. The range of variants in *TG* included missense ($n = 5$), nonsense ($n = 1$), and splicing ($n = 1$) (Table 1). In total, variants in *SLC5A5*, *SLC26A4* and *IYD* genes were detected in 10 patients (10.9%) (Table 1).

Frequency distribution of variants in genes associated with TD was as follows: *TSHR* 6.5% (6/92), *NKX2-1* 2.2% (2/92), *NKX2-5* 1.1% (1/92), *PAX8* 3.3% (3/92). In our study, a deletion with frameshift and 4 missense variants were detected in *TSHR* (Table 2). MLPA was conducted in the patients with a single heterozygous variant (N71, N72-1, N72-2) and showed no extended deletions or insertions. We found one deletion with frameshift and one missense variant in *NKX2-1* gene and one heterozygous missense variant in *NKX2-5* gene (Table 2). In three cases mutations in *PAX8* were detected (Table 2), 2 of which were missense variants, and

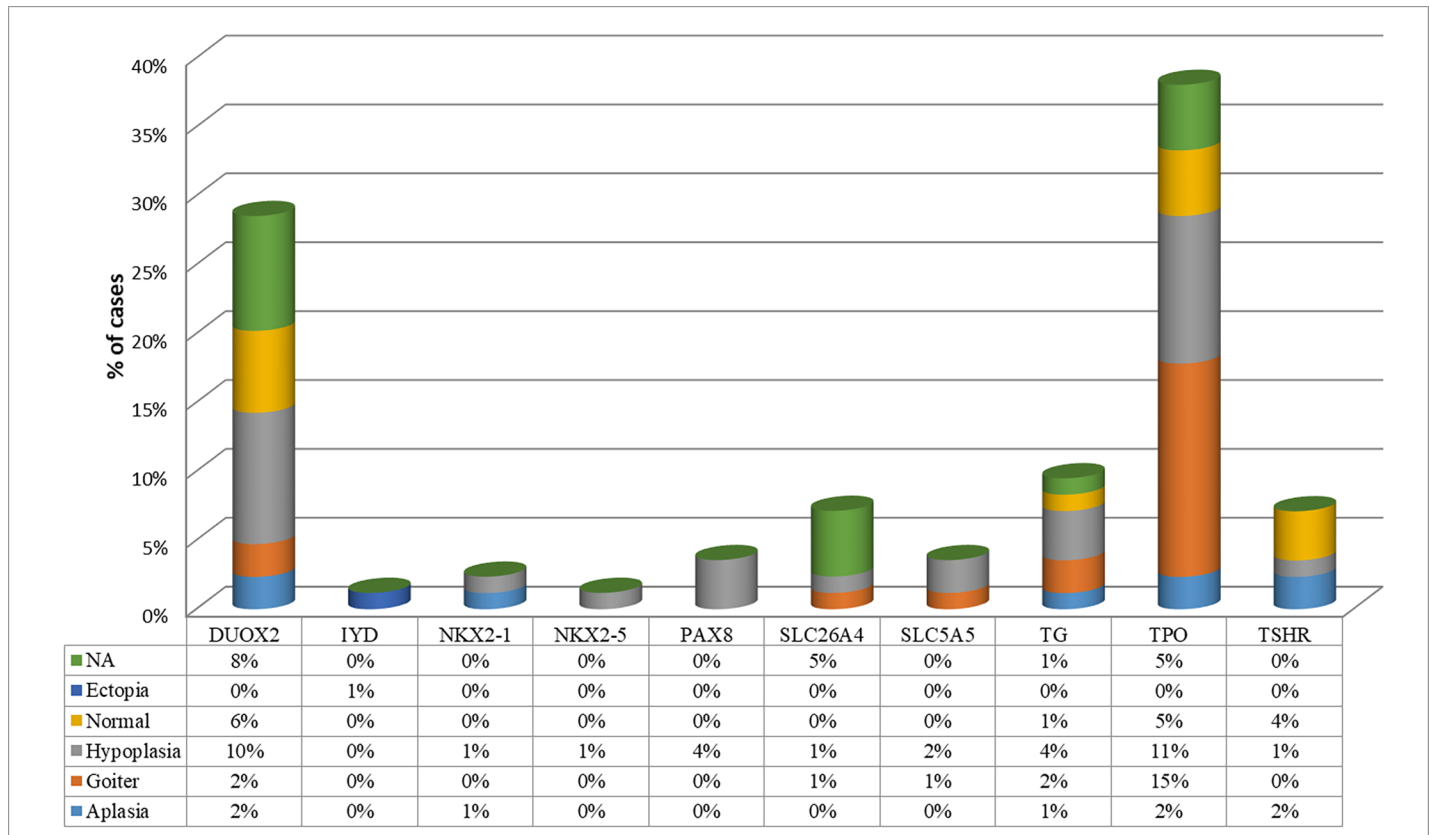


Fig 1. Percent distribution of monogenic variants identified in genes according to the CH phenotype.

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in one patient (N79) an extended deletion in the gene was suspected by NGS and subsequently confirmed by MLPA. There were no mutations in *DOUXA2* or *FOXE1* genes.

Mutations in two genes were revealed in 8 patients (8.7%) (Table 3). The most frequent combinations of variants in DH genes were *TG* and *TPO* (3 cases). 2 cases showed a combination of variants in DH and TD genes: *TG* and *PAX8* (1 case), and *TSHR* and *DUOX2* in 1 case.

In the control group, 4 heterozygous missense variants (7.1%) with uncertain significance were identified (Table 4). In comparison with the control group, the mutation rate in patients with CH was significantly higher (Pearson’s χ^2 ($p < 0.01$), odds ratio = 7.9, confidence interval 2.7–22.6).

In the group of patients with variants in one of DH genes, the most frequent pattern according to the ultrasound was hypoplasia of the thyroid gland, 32.0% (23/72), different forms of goiter, including multinodular, were identified in 26.4% (19/72), 11.1% of cases (8/72) had normal volume of the thyroid according to WHO criteria [32,33]. Thyroid aplasia was revealed in 6.9% (5/72) and ectopia in 1.4% (1/72). We were unable to obtain data of the thyroid size in 22.2% (16/72) of cases. The majority of cases with variants in TD genes showed hypoplasia or aplasia of the thyroid gland (75.0%, 9/12), three patients with variants in *TSHR* gene had normal thyroid volume.

Discussion

Recent studies have shown that the frequency of gene defects associated with CH is substantially higher than previously estimated, and ranges from 33,0% to 61,5% [24–28]. However,

Table 1. Summary of nucleotide variants in DH genes, characteristics and clinical manifestations.

Subjects	Gene	NT alteration	AA alteration	Pathogenicity	Zygoty	ExAC*	gnomAD^	HGMD#	Thyroid gland	Associated abnormalities
N1	TPO	c.1181_1182insCGGC	p.A397PfsX76	P	Het	NA	0.000523	NA	hypoplasia	None
N2	TPO	c.1181_1182insCGGC	p.A397PfsX76	P	Het	NA	0.000523	NA	goiter	None
N3	TPO	c.1181_1182insCGGC	p.A397PfsX76	P	Het	NA	0.000523	NA	multinodular goiter	None
N4	TPO	c.1181_1182insCGGC	p.A397PfsX76	P	Het	NA	0.000523	NA	NA	None
N5	TPO	c.1181_1182insCGGC	p.A397PfsX76	P	Het	NA	0.000523	NA	goiter	None
N6	TPO	c.1851delC	p.S617RfsX23	P	Het	NA	NA	NA	goiter	None
N7	TPO	c.2618+1G>T		P	Het	NA	NA	NA	hypoplasia	None
N8	TPO	c.A1898T	p.D633V	US	Het	NA	NA	NA	goiter	None
N9	TPO	c.C1449A	p.N483K	US	Het	NA	NA	NA	aplasia	None
N10	TPO	c.C265T	p.R89X	P	Het	NA	0.000008	Reported [43]	hypoplasia	None
N11	TPO	c.C443T	p.A148V	US	Het	0.000049	0.000043	NA	aplasia	None
N12	TPO	c.G1581T	p.W527C	LP	Het	NA	0.000069	Reported [22]	goiter	None
N13	TPO	c.G1751A	p.R584Q	US	Het	0.000082	0.000072	NA	hypoplasia	None
N14	TPO	c.G1994A	p.R665Q	LP	Het	0.000025	0.000024	Reported [44]	goiter	None
N15	TPO	c.G2017A	p.E673K	US	Het	0.00011	0.00009	NA	hypoplasia	None
N16	TPO	c.G2017A	p.E673K	US	Het	0.00011	0.00009	NA	hypoplasia	None
N17	TPO	c.G2017A	p.E673K	US	Het	0.00011	0.00009	NA	NA	None
N18	TPO	c.G2017A	p.E673K	US	Het	0.00011	0.00009	NA	NA	None
N19	TPO	c.T289C	p.S97P	US	Het	NA	NA	NA	goiter	None
N20	TPO	c.C208G	p.P70A	US	Het	0.00071	0.00086	NA	Normal	None
N21	TPO	c.T289C	p.S97P	US	Het	NA	NA	NA	hypoplasia	None
N22-1	TPO	c.G1042A	p.G348R	US	ComHet	NA	NA	NA	hypoplasia	None
	TPO	c.G1465A	p.A489T	US		NA	0.000037	NA		
N22-2	TPO	c.G1042A	p.G348R	US	ComHet	NA	NA	NA	hypoplasia	None
	TPO	c.G1465A	p.A489T	US		NA	0.000037	NA		
N23	TPO	c.1851delC	p.S617RfsX23	P	ComHet	NA	NA	NA	Normal	None
	TPO	c.2422delT	p.C808AfsX24	P		NA	0.000016	NA		
N24	TPO	c.2422delT	p.C808AfsX24	P	ComHet	NA	0.000016	NA	NA	None
	TPO	c.C208G	p.P70A	US		0.00071	0.00086	NA		
N25	TPO	c.C265T	p.R89X	P	ComHet	NA	0.000008	Reported [43]	multinodular goiter	Sensorineural hearing loss
	TPO	c.1181_1182insCGGC	p.A397PfsX76	P		NA	0.000523	NA		
N26	TPO	c.T391C	p.S131P	LP	ComHet	0.000058	0.000049	Reported [45]	multinodular goiter	None
	TPO	c.2386+2T>G		LP		NA	NA	NA		
N27-1	TPO	c.667_669delGAT	p.D223del	P	ComHet	NA	NA	NA	goiter	None

(Continued)

Table 1. (Continued)

Subjects	Gene	NT alteration	AA alteration	Pathogenicity	Zygoty	ExAC*	gnomAD^	HGMD#	Thyroid gland	Associated abnormalities
	<i>TPO</i>	c.2422delT	p.C808AfsX24	P		NA	0.000016	NA		
N27-2	<i>TPO</i>	c.667_669delGAT	p.D223del	P	ComHet	NA	NA	NA	goiter	None
	<i>TPO</i>	c.2422delT	p.C808AfsX24	P		NA	0.000016	NA		
N28	<i>TPO</i>	c.T281C	p.M94T	US	ComHet	NA	0.000007	NA	goiter	None
	<i>TPO</i>	c.A719T	p.D240V	US		NA	NA	NA		
N29	<i>DUOX2</i>	c.2895_2898del	p.S965fsX30	P	Het	0.0029	NA	Reported [46]	hypoplasia	None
N30	<i>DUOX2</i>	c.2895_2898del	p.S965fsX30	P	Hom	0.0029	NA	Reported [46]	goiter	None
N31	<i>DUOX2</i>	c.2895_2898del	p.S965fsX30	P	Het	0.0029	NA	Reported [46]	hypoplasia	None
N32	<i>DUOX2</i>	c.2895_2898del	p.S965fsX30	P	Het	0.0029	NA	Reported [46]	aplasia	None
N33	<i>DUOX2</i>	c.2895_2898del	p.S965fsX30	P	Hom	0.0029	NA	Reported [46]	NA	None
N34	<i>DUOX2</i>	c.2895_2898del	p.S965fsX30	P	Het	0.0029	NA	Reported [46]	hypoplasia	None
N35	<i>DUOX2</i>	c.2895_2898del	p.S965fsX30	P	Het	0.0029	NA	Reported [46]	Normal	None
N36	<i>DUOX2</i>	c.2895_2898del	p.S965fsX30	P	Hom	0.0029	NA	Reported [46]	hypoplasia	None
N37	<i>DUOX2</i>	c.2895_2898del	p.S965fsX30	P	Hom	0.0029	NA	Reported [46]	goiter	None
N38	<i>DUOX2</i>	c.2895_2898del	p.S965fsX30	P	Hom	0.0029	NA	Reported [46]	Normal	None
N39	<i>DUOX2</i>	c.2895_2898del	p.S965fsX30	P	Hom	0.0029	NA	Reported [46]	Normal	None
N40	<i>DUOX2</i>	c.2895_2898del	p.S965fsX30	P	Het	0.0029	NA	Reported [46]	hypoplasia	None
N41	<i>DUOX2</i>	c.2895_2898del	p.S965fsX30	P	Hom	0.0029	NA	Reported [46]	NA	None
N42	<i>DUOX2</i>	c.2895_2898del	p.S965fsX30	P	Het	0.0029	NA	Reported [46]	Normal	None
N43	<i>DUOX2</i>	c.2895_2898del	p.S965fsX30	P	Het	0.0029	NA	Reported [46]	Normal	None
N44	<i>DUOX2</i>	c.A4637G	p.E1546G	US	Het	0.00084	0.00081	NA	NA	None
N45	<i>DUOX2</i>	c.C1126T	p.R376W	US	Het	0.00012	0.00008	Reported [47]	aplasia	None
N46	<i>DUOX2</i>	c.C1294T	p.R432C	US	Het	NA	0.000004	NA	NA	None
N47	<i>DUOX2</i>	c.C3250T	p.R1084X	P	Het	0.000099	0.000087	NA	hypoplasia	None
N48	<i>DUOX2</i>	c.C3970T	p.P1324S	US	Het	NA	0.000008	NA	hypoplasia	None
N49	<i>DUOX2</i>	c.G1040A	p.R347K	US	Het	0.000034	0.000018	NA	NA	None
N50	<i>DUOX2</i>	c.A4637G	p.E1546G	US	Het	0.00084	0.00081	NA	hypoplasia	None
N51	<i>DUOX2</i>	c.T1366C	p.W456R	US	Het	NA	NA	NA	NA	None
N52	<i>DUOX2</i>	c.2895_2898del	p.S965fsX30	P	ComHet	NA	NA	Reported [46]	NA	None
	<i>DUOX2</i>	c.C2056T	p.Q686X	P		NA	0.000004	Reported [46]		
N53	<i>TG</i>	c.5401+2T>C		P	Het	NA	NA	NA	goiter	None

(Continued)

Table 1. (Continued)

Subjects	Gene	NT alteration	AA alteration	Pathogenicity	Zygoty	ExAC*	gnomAD^	HGMD#	Thyroid gland	Associated abnormalities
N54	TG	c.C2338A	p.Q780K	US	Het	NA	NA	NA	hypoplasia	None
N55	TG	c.G1900A	p.G634R	US	Het	0.00049	0.0005	NA	aplasia	None
N56	TG	c.G2776T	p.E926X	P	Het	NA	NA	NA	goiter	None
N57	TG	c.G2977A	p.A993T	US	Het	0.00033	0.00039	NA	Normal	None
N58	TG	c.G2977A	p.A993T	US	Het	0.00033	0.00039	NA	hypoplasia	None
N59	TG	c.T2200A	p.S734T	US	Het	0.000017	0.000022	NA	hypoplasia	None
N60	TG	c.G455A	p.R152H	US	Het	0.00068	0.00072	NA	NA	None
N61-1	SLC5A5	c.C1906T	p.R636X	P	Hom	NA	0.000011	NA	hypoplasia	None
N61-2	SLC5A5	c.C1906T	p.R636X	P	Hom	NA	0.000011	NA	hypoplasia	None
N62	SLC5A5	c.469delA	p.N157fs	P	ComHet	NA	NA	NA	goiter	None
	SLC5A5	c.G1183A	p.G395R	LP		0.000066	0.000047	Reported [48]		
N63	SLC26A4	c.A1246C	p.T416P	LP	Het	0.00021	0.0002	Reported [49]	NA	None
N64	SLC26A4	c.A736C	p.N246H	US	Het	0.0000082	0.000004	NA	NA	Sensorineural hearing loss
N65	SLC26A4	c.G1483A	p.D495N	US	Het	NA	NA	NA	hypoplasia	None
N66	SLC26A4	c.G441A	p.M147I	US	Het	0.00051	0.0006060	Reported [50]	NA	None
N67	SLC26A4	c.G441A	p.M147I	US	Het	0.00051	0.0006060	Reported [50]	goiter	None
N68	SLC26A4	c.G2219T	p.G740V	US	Het	0.00027	0.00029	Reported [51]	NA	None

(Continued)

these studies were limited by either the number of genes selected for analysis [27,28] or the number of the patients included in the study [24,25,27]. In addition, relatively soft filtering criteria for selection of pathogenic variants have been reported, allowing for MAF as high as 0.01 [24,26–28]. In the current study, using an NGS panel for 12 CH genes associated both with thyroid dysgenesis and dyshormonogenesis disorders, we have assessed the spectrum of gene defects in Russian subjects with severe CH, regardless of the thyroid anatomy findings. We have used more stringent criteria for selection of potentially pathogenic sequence variants, which were based on the recent ACMG guidelines [29]. As the result, from the analysis were excluded all single nucleotide variants with MAF greater than 0.001. For instance, P303R variant in *DUOX2* gene (rs151261408, MAF = 0.01), rated as likely pathogenic by Lof et al [24], was found in our cohort in 24 of 243 subjects (not shown). This variant previously shown to have no effect on *DUOX2* function by *in vitro* experiments [34] is classified as BS1, BS3 (benign) by ACMG rating [29] and excluded from analysis.

The results of the study demonstrate the genetic heterogeneity of CH and a high incidence of cases with pathogenic or potentially pathogenic variants in one of the CH candidate genes (37.9%), both in patients with thyroid dysgenesis and goiter and normal size of the gland. In

Table 2. Summary of nucleotide variants in TD genes, characteristics and clinical manifestations.

Subjects	Gene	NT alteration	AA alteration	Pathogenicity	Zygoty	ExAC*	gnomAD^	HGMD#	Thyroid gland	Associated abnormalities
N70-1	<i>TSHR</i>	c.141delC	p.I47fs	P	Hom	NA	NA	NA	aplasia	None
N70-2	<i>TSHR</i>	c.141delC	p.I47fs	P	Hom	NA	NA	NA	aplasia	None
N71	<i>TSHR</i>	c.C484G	p.P162A	LP	Het	0.00017	0.0001371	Reported [53]	hypoplasia	None
N72-1	<i>TSHR</i>	c.G902A	p.C301Y	US	Het	NA	0.000032	NA	Normal	None
N72-2	<i>TSHR</i>	c.G902A	p.C301Y	US	Het	NA	0.000032	NA	Normal	None
N73	<i>TSHR</i>	c.C1532T	p.T511M	US	ComHet	0.000033	0.000033	NA	Normal	None
	<i>TSHR</i>	c.T1697G	p.V566G	US		NA	NA	NA		
N74	<i>NKX2-1</i>	c.628_772del		P	Het	NA	NA	NA	hypoplasia	chorea
N75	<i>NKX2-1</i>	c.A1180G	p.T394A	US	Het	NA	NA	NA	aplasia	None
N76	<i>NKX2-5</i>	c.G676A	p.D226N	US	Het	NA	NA	NA	hypoplasia	None
N77	<i>PAX8</i>	c.A701G	p.E234G	US	Het	NA	0.000037	NA	hypoplasia	None
N78	<i>PAX8</i>	c.G440A	p.C147Y	US	Het	NA	NA	NA	hypoplasia	None
N79	<i>PAX8</i>	chr2:113973574_114036498del		P	Het	NA	NA	NA	hypoplasia	None

The Human Gene Mutation Database (HGMD®) (<http://www.hgmd.cf.ac.uk>) [52]

*ExAC database (<http://exac.broadinstitute.org>) [36]

^gnomAD database (<http://gnomad.broadinstitute.org/>)

Pathogenicity: US, Uncertain significance; P, Pathogenic; LP, Likely pathogenic (pathogenicity rated according to ACMG guidelines [29], sequence variants rated as 'benign' or 'likely benign' were excluded from the analysis); NT, nucleotide; AA, amino acid; NA, not available; Het, heterozygous; ComHet, compound heterozygous; Hom, homozygous.

NCBI Reference Sequences (www.ncbi.nlm.nih.gov/nucore): *TSHR*, NM_000369; *NKX2-1*, NM_001079668; *NKX2-5*, NM_004387; *PAX8*, NM_003466.

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general, according to Exome Aggregation Consortium (ExAC) data (<http://exac.broadinstitute.org/>), the majority of CH genes (DH genes, in particular) show higher than expected variant counts (low intolerance to variation) [35]. To evaluate the chances of having a variant in one of CH genes in subjects without CH we have sequenced the candidate genes in 56 subjects with normal thyroid function and demonstrated a significantly lower rate of variants compared to the CH group (OR = 7.9, p<0.01).

Moreover, according to the results of our study, the most frequent findings in severe CH were variants in DH genes 84.8% (78/92), while only 13.1% (12/92) of cases were associated with variants in TD genes, which contradicts to the expected distribution of etiological forms based on the results of ultrasound and scintigraphy [2,3]. The more prevalence of mutations in DH genes compared to TD genes have been also reported in other NGS-based studies [24,25,27,28].

The majority of TD disorders were originally described as autosomal recessive, however, a large proportion of variants identified in our study, both using targeted NGS and additional screening of extended deletions by MLPA, were heterozygous. Existence of additional mutation in non-coding regions of the studied genes can not be completely ruled out. Another possible explanation could be non-Mendelian mechanisms of inheritance, such as autosomal monoallelic expression (AME) [36–38]. Initially, autosomal monoallelic expression of the mutant allele was described for *TPO* gene [36]. The subsequent study by Magne et al. demonstrated AME on average for 22 genes [16–32] expresses in the thyroid [39]. Monoallelic mutations in TD genes in subjects with CH have been reported by others [25,26]. Fan et al. identified 9 cases with mutations in *TG* gene, all of which were heterozygous [25].

Table 3. Digenic mutations, characteristics and clinical manifestations.

Subjects	Gene	NT alteration	AA alteration	Pathogenicity	Zygoty	ExAC*	gnomAD^	HGMD#	Thyroid gland	Associated abnormalities
N80	<i>PAX8</i>	c.C74T	p.P25L	US	Het	NA	NA	NA	hypoplasia	None
N80	<i>TG</i>	c.C961T	p.R321X	P	Het	NA	NA	NA		
N81	<i>TG</i>	c.C6553T	p.R2185W	US	Het	NA	0.000048	NA	hypoplasia	None
N81	<i>TPO</i>	c.C208G	p.P70A	US	Het	0.00072	0.00086	NA		
N82	<i>IYD</i>	c.C818T	p.T273M	US	Het	0.00013	0.00011	NA	hypoplasia	None
N82	<i>TG</i>	c.G2977A	p.A993T	US	Het	0.00033	0.00039	NA		
N83	<i>DUOX2</i>	c.2895_2898del	p.S965fsX30	P	Het	0.0029	NA	Reported [46]	hypoplasia	None
N83	<i>TSHR</i>	c.G733A	p.G245S	US	Het	0.00014	0.00009	Reported [54]		
N84	<i>TG</i>	c.G455A	p.R152H	US	Het	0.00068	0.00073	NA	goiter	None
N84	<i>TPO</i>	c.C290G	p.S97X	P	Het	NA	NA	NA		
N85	<i>DUOX2</i>	c.A4603G	p.R1535G	US	Het	0.00027	0.00029	NA	goiter	None
N85	<i>TPO</i>	c.C962T	p.T321I	US	Het	NA	NA	NA		
N86	<i>DUOX2</i>	c.2895_2898del	p.S965fsX30	P	Hom	0.0029	NA	Reported [46]	goiter	None
N86	<i>SLC26A4</i>	c.G441A	p.M147I	US	Het	0.00051	0.0006	Reported [50]		
N87	<i>TG</i>	c.C4481T	p.P1494L	US	Het	0.00054	0.00047	NA	goiter	None
N87	<i>TPO</i>	c.G1450A	p.V484M	US	Het	NA	NA	NA		

The Human Gene Mutation Database (HGMD®) (<http://www.hgmd.cf.ac.uk>) [52]

*ExAC database (<http://exac.broadinstitute.org>) [36]

^gnomAD database (<http://gnomad.broadinstitute.org/>)

Pathogenicity: US, Uncertain significance; P, Pathogenic; LP, Likely pathogenic (pathogenicity rated according to ACMG guidelines [29], sequence variants rated as 'benign' or 'likely benign' were excluded from the analysis); NT, nucleotide; AA, amino acid; NA, not available; Het, heterozygous; ComHet, compound heterozygous; Hom, homozygous.

NCBI Reference Sequences (www.ncbi.nlm.nih.gov/nucleotide): *TPO*, NM_000547; *DUOX2*, NM_014080; *TG*, NM_003235; *SLC26A4*, NM_000441; *IYD*, NM_203395; *TSHR*, NM_000369; *PAX8*, NM_003466.

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Another unexpected finding was the absence of goiter in some patients with defects in DH genes. A similar observation has been made by Kühnen et al. who detected a homozygous missense mutation in *SLC26A4* gene in patients with thyroid hypoplasia [40]. The authors suggested a role of severe postnatal iodine deficiency as a possible explanation of this phenomenon [41]. Another reason for the absence of enlargement of the thyroid can be anti-goitrogenic effect of levothyroxine.

Table 4. Control group.

Subjects	Gene	NT alteration	AA alteration	Pathogenicity	Zygoty	ExAC*	gnomAD^	HGMD#
C1	<i>DUOX2</i>	c.C4632G	p.H1544Q	US	Het	NA	NA	NA
C2	<i>IYD</i>	c.A281G	p.Y94C	US	Het	0.000025	0.00004	NA
C3	<i>SLC26A4</i>	c.C1232G	p.A411G	US	Het	NA	NA	NA
C4	<i>TG</i>	c.A6853G	p.N2285D	US	Het	NA	NA	NA

The Human Gene Mutation Database (HGMD®) (<http://www.hgmd.cf.ac.uk>) [52]

*ExAC database (<http://exac.broadinstitute.org>) [36]

^gnomAD database (<http://gnomad.broadinstitute.org/>)

Pathogenicity: US, Uncertain significance; P, Pathogenic; LP, Likely pathogenic (pathogenicity rated according to ACMG guidelines [29], sequence variants rated as 'benign' or 'likely benign' were excluded from the analysis); NT, nucleotide; AA, amino acid; NA, not available; Het, heterozygous; ComHet, compound heterozygous; Hom, homozygous.

NCBI Reference Sequences (www.ncbi.nlm.nih.gov/nucleotide): *DUOX2*, NM_014080; *TG*, NM_003235; *SLC26A4*, NM_000441; *IYD*, NM_203395.

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Similar to some previous reports [24–28,41], we identified patients with digenic mutations. The development of hypothyroidism in such cases is explained by synergistic heterozygosity, so the presence of heterozygous mutations in several genes can lead to cross-loss of enzyme activity [42]. In our study digenic mutations were found in 8 patients. Interestingly, goiter in this group was identified only in patients with 2 mutations of DH genes, while patients with mutations both in DH and TD genes showed a decrease in the volume of the gland.

In summary, a targeted next generation sequencing in patients with severe CH revealed potentially pathogenic sequence variants in more than a third of the cases, with a preponderance of those in genes associated with thyroid dysmorphogenesis.

Supporting information

S1 Fig. Sanger confirmation of sequence variants identified by NGS. A) *TPO* c.1181_1182 insCGGC; B) *TPO* c.1851delC; C) *TPO* c.G1581T; D) *TPO* c.G1994A; E) *TPO* c.G2017A; F) *TPO* c.G1042A; G) *TPO* c.667_669delGAT; H) *TPO* c.2422delT; I) *TPO* c.A719T; J) *DUOX2* c.2895_2898del; K) *DUOX2* c.A4637G.

(TIF)

S2 Fig. Sanger confirmation of sequence variants identified by NGS. A) *DUOX2* c.C1126T; B) *DUOX2* c.C1294T; C) *DUOX2* c.C3250T; D) *DUOX2* c.C3970T; E) *DUOX2* c.G1040A; F) *DUOX2* c.T1366C; G) *TG* c.C2338A; H) *TG* c.G2977A; I) *SLC5A5* c.C1906T; J) *SLC5A5* c.469delA; K) *SLC26A4* c.A736C; L) *SLC26A4* c.G441A.

(TIF)

S3 Fig. Sanger confirmation of sequence variants identified by NGS. A) *SLC26A4* c.G2219T; B) *IYD* c.C448T; C) *TSHR* c.141delC; D) *TSHR* c.C484G; E) *TSHR* c.G902A; F) *TSHR* c.C1532T; G) *NKX2-1* c.628_772del; H) *NKX2-1* c.A1180G; I) *NKX2-5* c.G676A; J) *PAX8* c.A701G; K) *PAX8* c.G440A; L) *PAX8* c.C74T.

(TIF)

S4 Fig. Sanger confirmation of sequence variants identified by NGS. A) *TG* c.C961T; B) *TG* c.C6553T; C) *TSHR* c.G733A; D) *TG* c.G455A; E) *DUOX2* c.A4603G; F) *TG* c.C4481T; G) *TPO* c.G1450A; H) *TPO* c.C443T; I) *TPO* c.T391C.

(TIF)

S5 Fig. MLPA result. *PAX8* chr2:113973574_114036498del.

(TIFF)

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