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Chromosomal microarray and whole exome sequencing identify genetic causes of congenital hypothyroidism with extra-thyroidal congenital malformations

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ABSTRACT

Background: Congenital hypothyroidism (CH) is the most common neonatal endocrine disorder. Although most patients present with isolated CH, some patients present with CH and extra-thyroidal congenital malformations (ECMs), for which less is known about the underlying genetics. The aim of this study was to investigate the genetic mechanisms in patients with CH and ECMs using chromosomal microarray (CMA) and whole exome sequencing (WES).

Methods: Peripheral venous blood samples were collected from 16 patients with CH and ECMs. Genomic DNA was extracted from peripheral blood leukocytes. CMA and WES were performed to detect copy number and single nucleotide variants.

Results: CMA identified clinically significant copy number variants in 7 patients consistent with their phenotypes. For 6 of them, the genotype and phenotype suggested a syndromic diagnosis, and the remaining patient carried a pathogenic microdeletion and microduplication including GLIS3. WES analysis identified 9 different variants in 7 additional patients. The variants included 2 known mutations (c.1096C > T (p.Arg366Trp) in KCNQ1 and c.848C > A (p.Pro283Gln) in NKX2-5) and 7 novel variants: one nonsense mutation (c.4330C > T (p.Arg1444*) in ASXL3), one frameshift mutation (c.1253_1259delACTCTGG (p.Asp418fs) in TG), three missense variants (c.1472C > T (p.Thr491Ile) in TG, c.4604A > G (p.Asp1535Gly) in TG, and c.2139G > T (p.Glu713Asp) in DUOX2, and two splice site variants (c.944-1G > C and c.3693 + 1G > T) in DUOX2.

Conclusions: We report the first genetic study of CH patients with ECMs using CMA and WES. Overall, our detection rate for pathogenic and possibly pathogenic variants was 87.5% (14/16). We report 7 novel variants, expanding the mutational spectrum of TG, DUOX2, and ASXL3.

1. Introduction

Congenital hypothyroidism (CH) is the most common neonatal endocrine disorder in infancy, with prevalence ranging from 1:2000 to 1:4000 newborns [1,2]. Previously, we have found that CH is slightly more prevalent in Guangxi Zhuang Autonomous Region of China (1:1694) compared to worldwide levels, with 911 and 731 of 1,238,340 infants in the newborn screening program subsequently diagnosed with hyperthyrotropinemia (elevated TSH) and CH, respectively [3]. Sixty six of the 731 patients with CH were sequenced to detect CH-associated genes by CH capture panel, and 32 of them (48.5%) had at least one potential pathogenic variant [1].

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Multiple genes, including *FOXE1, GLIS3*, *NKX2.1*, *NKX2.5, PAX8, SLC26A4*, and *TSHR* have been associated with CH. The majority of these genes have been associated with isolated CH, and there have only been a few reports of genes associated with CH and extra-thyroidal congenital malformations (ECMs) [4–6]. For example, *SLC26A4* mutations can lead to CH with deafness [7] and *GLIS3* mutations can lead to a multi-system phenotype including CH and neonatal diabetes [8–11].

Recent studies have reported on the increased occurrence of ECMs in CH patients. Kumar et al. found a high percentage of additional congenital malformations (27% of 1538 patients with CH in their study population). Congenital renal and urological anomalies account for the most, followed by cardiac, gastrointestinal, and skeletal in the patients with CH [12]. In Iran, 32 of 100 permanent CH had urogenital abnormalities [13]. Kreisner et al. detected malformations in 10 of 76 (13.2%) patients with permanent CH, predominantly cardiac defects, but cleft palate and lip and bifid spine were also found [14]. However, there is no study specifically examining the genetic causes of CH patients with ECMs using CMA and WES. Given the rarity of this condition that the genotype-phenotype relationship has not yet been fully established, little is known about its genetic mechanisms and prevalence among Chinese CH patients with ECMs. Here, we investigate the genetic mechanisms in 16 patients with CH and ECMs by CMA and WES.

2. Methods

2.1. Patients

We enrolled 16 patients with CH and ECMs in this study. The patients were identified by newborn screening in the Maternal and Child Health Hospital of Guangxi Zhuang Autonomous Region. The methods of newborn screening for CH have been described in detail previously [1,2]. The study was approved by the local Medical Ethics Committee. Informed consent was obtained from the parents of the patients.

2.2. CMA and WES analysis and validation

Peripheral venous blood samples were collected from the patients. Genomic DNA was extracted from peripheral blood leukocytes using QIAamp DNA Blood Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. CMA [15] and WES [16] were performed to detect copy number variations (CNVs) and single nucleotide variations (SNVs), respectively, as described in detail previously. Sanger sequencing was used to validate mutations identified from WES.

3. Results

3.1. Chromosome microarray analysis

CMA identified clinically significant CNVs in seven patients, which were consistent with the clinical phenotypes (Fig. 1 and Table 1). For six of these patients, the genotype and phenotype suggested a syndromic diagnosis that can lead to CH and ECMs, including Williams-Beuren syndrome (Fig. 1A), 2q37.3 deletion syndrome (Fig. 1B-C), Down syndrome (Fig. 1D), Warkany syndrome (Fig. 1G), 18p11.3 deletion syndrome (Fig. 1H–I), and Prader-Willi/Angleman syndrome (Fig. 1J). The remaining patient, Patient 4, carries a chromosomal 7p22.3~p21.3 duplication (Fig. 1E) and 9p24.3~p23 deletion (Fig. 1F), and the deletion region include *GLIS*3 and other disease-causing genes, leading to the multisystem phenotype including CH.

3.2. Whole exome sequencing analysis

WES analysis identified nine variants in seven additional patients, their phenotypic presentations and genetic variants are described in Tables 2 and 3. All variants were confirmed by Sanger sequencing (**Fig. S1**). The variants included two known mutations, c.1096C > T (p.Arg366Trp) (HGMD CM981130) in *KCNQ1* and c.848C > A (p.Pro283Gln) (HGMD CM108634) in *NKX2.5*. In addition, we report 7 novel variants: one nonsense mutation c.4330C > T (p.Arg1444*) in *ASXL3*, one frame shift mutation c.1253_1259delACTCTGG (p.Asp418fs) in *TG*, three missense variants c.1472C > T (p.Thr491Ile) and c.4604A > G (p.Asp1535Gly) in *TG* and c.2139G > T (p.Glu713Asp) in *DUOX2*, and two splice site variants c.944-1G > C and c.3693 + 1G > T in *DUOX2*.

Of these nine variants, the two truncating variants c.4330C > T (p.Arg1444*) in *ASXL3* and c.1253_1259delACTCTGG (p.Asp418fs) in *TG*, and the two splice site variants c.944-1G > C and c.3693 + 1G > T in *DUOX2* are classified as pathogenic or likely pathogenic according to ACMG/AMP guidelines [17]. The remaining five variants were evaluated and classified as variant of unknown significance (VUS) as they currently lack sufficient evidence to support pathogenicity according to ACMG/AMP guidelines. Of the nine variants, seven variants in mutant genes (*TG*, *DUOX2*, and *NKX2-5*) are associated with CH. For the remaining two variants, one in gene *ASXL3* and the other one in *KCNQ1* is associated with Bainbridge-Ropers syndrome and Long QT syndrome, respectively. Among these seven patients, the candidate variants were confirmed or partially confirmed to their clinical phenotypes.

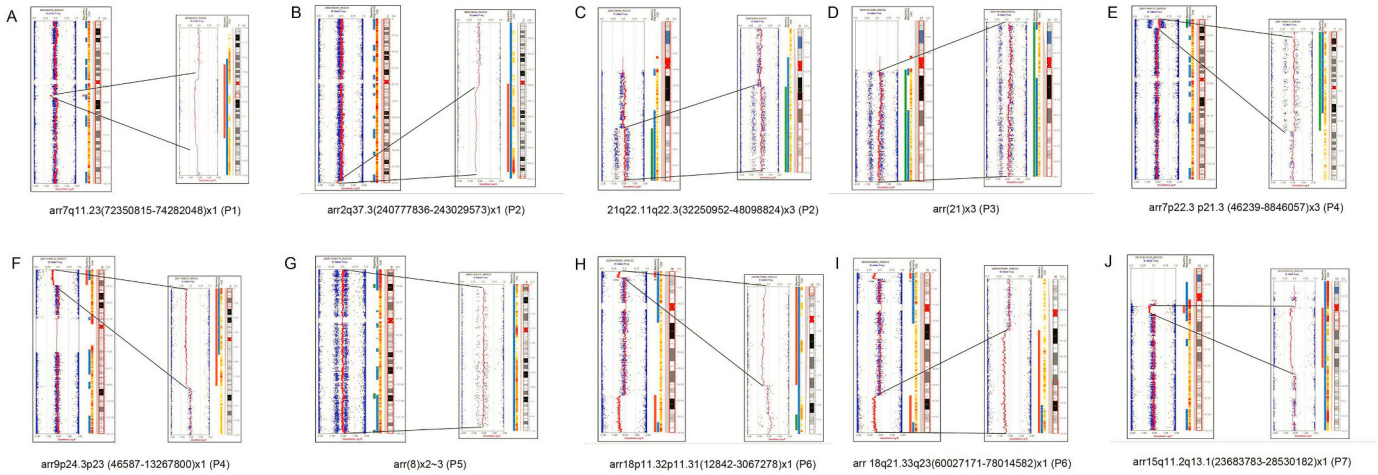

Fig. 1. CMA testing results for CH patients with ECMs and detected pathogenic CNVs. The subpanels show CNVs detected in (A) Patient 1, (B–C) Patient 2, (D) Patient 3, (E–F) Patient 4, (G) Patient 5, (H–I) Patient 6, and (J) Patient 7.

Table 1
Clinical and genetic information for 7 CH patients with ECMs and pathogenic CNVs.

ID#	Sex	Age	Phenotypes	Detected CNVs	Syndrome/causative genes	Comorbidities associated with syndrome/causative genes
1	M	3 mo	CH, low set ears, left hand with simian line, narrow jaw, developmental delay	arr 7q11.23(72350815-74282048)x1	Williams-Beuren Syndrome	CH, developmental delay, special face, umbilical hernia, growth retardation, congenital heart disease, sensorineural deafness, skeletal deformities
2	M	3 mo	CH, developmental & growth delay, poor response, gold fish eye, hand with simian line, congenital heart disease, micrognathia	arr 2q37.3(240777836-243029573)x1, 21q22.11q22.3(32250952-48098824)x3	2q37.3 Deletion Syndrome	CH, distinctive facies, congenital heart disease, obesity, round face, intellectual disability, a specific cognitive profile, unique personality characteristics, growth abnormalities, behavioral problems
3	NA	NA	CH, atrial septal defect, growth delay	arr (21)x3	Down syndrome	CH, congenital heart disease, short stature, obesity, round face, intellectual disability
4	F	9 yr	CH, mental retardation, language development delay, flat nose bridge, round nose, low ear position, low hairline, short neck, double toe	arr 7p22.3 p21.3 (46239-8846057)x3; arr 9p24.3p23 (46587-13267800)x1	*GLIS3*	CH, global developmental delay, abnormal facial shape, seizures, intellectual disability, and/or other significant developmental or morphological phenotypes
5	M	14 mo	CH, growth delay, difficult to stand, microcephaly, language development delay	arr (8)x2~3	Warkany syndrome	CH, facial dysmorphism, developmental delay
6	M	4 mo	CH, right clubfoot varus, atrial septal defect	arr 18p11.32p11.31(12842-3067278)x1; arr 18q21.33q23(60027171-78014582)x1	18p11.3 deletion syndrome	CH, congenital heart defects, muscular hypotonia, cleft palate, scoliosis, intellectual disability, microcephaly, developmental delay and/or other significant developmental or morphological phenotypes
7	M	7 yr	CH, mental retardation, short stature, hypophysis dysplasia, hypotonia	arr 15q11.2q13.1(23683783-28530182)x1	Prader-Willi syndrome (PWS)/Angleman Syndrome (AS)	CH, muscular hypotonia, cleft palate, intellectual disability, microcephaly, developmental delay and/or other significant developmental or morphological phenotypes

CH: congenital hypothyroidism; ECMs: extra-thyroidal congenital malformations; CNVs: copy number variations; M: male; F: female; mo: months; yr: years.

3.3. CMA and WES negative cases

Two patients with CH and ECMs had negative results from both CMA and WES. The first patient presented with short stature, intellectual disability, and autism spectrum disorder; and the second patient presented with short stature, pectus carinatum, and development delay, especially for motor milestones.

4. Discussion

The majority of CH studies have focused on isolated CH, and thus the etiology of CH with ECMs remains to be fully elucidated. CH with ECMs may be caused by multiple mechanisms: 1) syndromes leading to CH and multi-system phenotypes, such as Down syndrome and Williams-Beuren Syndrome [18,19]; 2) single gene mutations, such as mutations in *GLIS3* and *SLC26A4* [7,10,11]; 3) multiple gene mutations (including CH-associated genes) [20]; and 4) non-genetic factors, such as iodine deficiency, drugs, and maternal influences during pregnancy (smoking, alcohol consumption, drug abuse, or other factors) [21]. To the best of our knowledge, this is the first study to investigate the genetic mechanisms in CH patients with ECMs using CMA and WES. In our cohort of 16 CH patients with ECMs, we detected pathogenic or likely pathogenic variants in 87.5% (14/16) of patients, specifically pathogenic CNVs in seven patients (7/16, 43.8%), and pathogenic or likely pathogenic SNVs in another seven patients (7/16, 43.8%). Thus, we demonstrate that CMA and WES together provide an excellent genetic detection rate for CH patients with ECMs. For the two patients with negative results from CMA and WES, the underlying cause may be genetic factors not able to be detected by CMA or WES, such as intronic mutations or partial gene deletions, or non-genetic factors.

Of the seven CH patients with ECMs and detected pathogenic CNVs, six patients were diagnosed with specific syndromes, which are known to be associated with CH. However, the mechanisms leading to specific syndromes in these CH patients remain unknown. Delayed maturation of the hypothalamic–pituitary–thyroid axis has been proposed as the cause of thyroid dysfunction in patients with Williams-Beuren Syndrome [22]. A similar pathogenetic mechanism has been proposed for thyroid disorders observed in patients with Down syndrome [18]. Another study suggests that there is a link between oxidative stress and thyroid dysfunction in Down syndrome [23]. In summary, further studies are required to find the potential link between thyroid disorders and pathogenetic mechanism in syndromes.

It should be noted that some newborns with syndromes show signs and symptoms which can simulate CH. For example, Patient 3 presented with an atrial septal defect and failure to thrive, and Patient 7 presented with pituitarydysplasia, short stature, intellectual disability, and hypotonia. Both patients presented with signs and symptoms that can simulate CH and using CMA were diagnosed with Down syndrome and Prader-Willi syndrome, respectively. Thus, pediatricians should be aware that a patient found to have CH with ECMs who does not improve with adequate thyroid replacement therapy needs to be further investigated, including with genetic testing, for an additional diagnosis.

Of the seven CH patients with ECMs and detected possibly pathogenic variants, patient 14 presented with growth retardation, micrognathia, and congenital heart disease (atrial and ventricular septal defects), and carried a known heterozygous mutation, c.1096C > T (p.Arg366Trp), in *KCNQ1*. Gain-of-function mutations in *KCNQ1* have been reported to lead to short QT syndrome (SQTS) and lethal arrhythmias with autosomal dominant inheritance [24,25]. The KCNQ1 α subunit and the KCNE2 β subunit form a potassium channel in thyroid epithelial cells. Interestingly, genetic disruption of KCNQ1-KCNE2 causes hypothyroidism in mice, resulting in cardiac hypertrophy, dwarfism, alopecia, and prenatal mortality [26]. In addition, Patient 8 presented with global developmental delay and hypotonia, and carries a heterozygous mutation, c.4330C > T (p.Arg1444*) in *ASXL3* as well as a heterozygous mutation, c.4604A > G (p.Asp1535Gly) in *TG*.

Table 2
Clinical and genetic information for 7 CH patients with ECMs and SNVs and 2 patients with CH and ECMs and negative testing.

ID	Gender	Age	Clinical phenotypes	Gene	Inheritance	Exon	Position	Variant(s)	Zygosity	Pathogenicity (ACMG/AMP)	Novelty	Origin	OMIM	Clinical fit
8	F	7 mo	CH	*TG*	AR	22/48	Chr 8:133935658	NM_003235.4: c.4604A > G/p.Asp1535Gly	Het	VUS	ExAC: 12	Mat	#274700; Thyroid dyshormonogenesis 3; TDH3	Yes but inconsistency of inheritance mode
			Developmental delay, hypotonia	*ASXL3*	AD	12/12	Chr 18:31324142	NM_030632.2: c.4330C > T/p.Arg1444*	Het	P	Previously reported	Presumed de novo; father sample not available	Bainbridge-Ropers syndrome	Yes
9	F	1 yr	CH	*DUOX2*	AR	17/34	Chr 15:45398332	NM_014080.4: c.2139G > T/p.Glu713Asp	Het	VUS	Novel	Pat	#607200; Thyroid dyshormonogenesis 6;	Yes
						splicing, intron8-exon9	Chr 15:45402723	NM_014080.4: c.944-1G > C	Het	LP	Novel	Mat	TDH6	
			Microcephaly, atresia auris, short stature	NK										
10	M	1 yr 2 mo	CH	*DUOX2*	AR	splicing, exon 28-intron 28	Chr 15:45389811	NM_014080.4: c.3693 + 1G > T	Hom	P	ExAC: 13	Pat & Mat	# 607200; Thyroid dyshormonogenesis 6; TDH6	Yes
			Polydactyly	NK										
11	M	4 mo	CH, developmental delay, hypotonia, prominent forehead	*NKX2-5*	AD	2/2	Chr 5:172659699	NM_004387.3: c.848C > A/p.Pro283Gln	Het	VUS	ExAC: 7	Mat	#225250; Hypothyroidism, congenital nongoitrous, 5; CHNG5	Yes but considered VUS
12	F	3 mo	CH	*TG*	AR	9/48	Chr 8:133898866	NM_003235.4: c.1253_1259delACTCTGG/ p.Asp418fs	Het	P	Novel	Pat	#274700; Thyroid dyshormonogenesis 3; TDH3	Yes but inconsistency of inheritance mode
			Microcephaly, development delay	NK										
13	F	2 mo	CH	*TG*	AR	9/48	Chr 8:133899089	NM_003235.4: c.1472C > T/p.Thr491Ile	Het	VUS	ExAC: 3	NK	#274700; Thyroid dyshormonogenesis 3; TDH3	Yes but inconsistency of inheritance mode
			Large tongue, protruding eyes, skin pigmentation, clitoris hypertrophy	NK										
14	M	4 mo	Transient CH	*KCNQ1*	AD	8/16	Chr 11:2606505	NM_000218.2: c.1096C > T/p.Arg366Trp	Het	VUS	Previously reported	Pat	# 192500;Long QT syndrome 1; LQT1	Secondary finding but disruption of KCNQ1-KCNE2 may cause hypothyroidism in mice
			Growth retardation, micrognathia, congenital heart disease (ASD, VSD)	NK										

(*continued on next page*)

Table 2 (*continued*)

ID	Gender	Age	Clinical phenotypes	Gene	Inheritance	Exon	Position	Variant(s)	Zygosity	Pathogenicity (ACMG/AMP)	Novelty	Origin	OMIM	Clinical fit
15	M	3 mo 14 d	CH, mental retardation, short stature, autism	NK										
16	M	1 yr 6 mo	CH, pectus carinatum, developmental delay especially motor milestones, short stature	NK										

CH: congenital hypothyroidism; ECMs: extra-thyroidal congenital malformations; SNVs: single nucleotide variations; M: male; F: female; mo: months; yr: years; AD: autosomal dominant; AR: autosomal recessive; d: days; LP: likely pathogenic; mat: maternal; mo: months; NK: not known; P: pathogenic; pat: paternal; mat: maternal; ExAC:The Exome Aggregation Consortium database; VUS: variants of uncertain significance; Het: heterozygous; Hom: homozygous.

Table 3
Evaluation and classification of the nine genetic variants detected by WES.

Gene	Variant	Classification	PVS1	PM1	PM2		PM3	PP3/BP4	PP5
			Null variant in a gene where LOF is a known mechanism of disease	Located in functional domain	Frequency in globe Population (and East Asian)	Local frequency	Cis/trans testing	In-silico prediction (No. of algorithms predicted to deleterious/total) [citation]	Reported as pathogenic
ASXL3	p.Arg1444*	P	Nonsense	NA	0 (0)	0	NA	9/11	PMID: 26647312
DUOX2	c.944-1G > C	LP	Splice site acceptor	NA	0 (0)	0	Yes	NA	No
DUOX2	p.Glu713Asp	VUS	No	NA	0 (0)	0	Yes	8/23	No
DUOX2	c.3693 + 1G > T	P	Splice site donor	Ferric oxidoreductase	0.011% (0.15%)	0.15%	NA	NA	No
KCNQ1	p.Arg366Trp	VUS	No	Ion transport domain	0 (0)	0	NA	16/23	ClinVar: RCV000045959.5; RCV000057551.3
NKX2-5	p.Pro283Gln	VUS	No	NA	0.0064% (0.05%)	0.15%	NA	11/23	ClinVar: RCV000023023.2, associated with Ventricular septal defect 3 (VSD3)
TG	p.Asp418fs	P	Frameshift	TG type I-10	0 (0)	0	NA	NA	No
TG	p.Thr491Ile	VUS	No	NA	0.0025% (0.035%)	0	NA	8/23	No
TG	p.Asp1535Gly	VUS	No	TG type I-11	0.0099% (0.14%)	0.37%	NA	15/23	No

Classification and evidence system according to ACMG/AMP variants interpretation guidelines [17]
PVS1 = null variant (nonsense, frameshift, canonical ± 2 splice sites, initiation codon, single or multiexon deletion) in a gene where LOF is a known mechanism of disease
PM1 = Located in a mutational hot spot and/or critical and well-established functional domain (e.g., active site of an enzyme) without benign variation
PM2 = Absent from controls (or at extremely low frequency if recessive) in Exome Sequencing Project, 1000 Genomes, or Exome Aggregation Consortium
PM3 = For recessive disorders, detected in trans with a pathogenic variant
PP3 = Multiple lines of computational evidence support a deleterious effect on the gene or gene product (conservation, evolutionary, splicing impact, etc.)
PP5 = Reputable source recently reports variant as pathogenic, but the evidence is not available to the laboratory to perform an independent evaluation
BP4 = Multiple lines of computational evidence suggest no impact on gene or gene product (conservation, evolutionary, splicing impact, etc.)
P = Pathogenic; LP = likely pathogenic; VUS = Variants of Uncertain Significance; NA = not applicable.

Mutations in *ASXL3* have been identified in individuals with Bainbridge-Ropers Syndrome (BRS), characterized by failure to thrive, global developmental delay, feeding problems, hypotonia, dysmorphic features, profound speech delays and intellectual disability [27–29]. To date, 30 patients have been reported and de novo heterozygous truncating mutations have emerged as the cause of BRS [30]. The *ASXL3* mutation carried by Patient 8 was inherited from the father with a normal phenotype, suggesting incomplete penetrance.

In conclusion, it is important to note that CH can present with other congenital malformations. Recently, evidence of a genetic link between the incidence of CH and other congenital malformations has been proposed [31]. Based on this evidence, mutations in the genes *PAX8* (paired box 8), *FOXE1* (forkhead box E1), *TTF1* (thyroid transcription factor 1), *TTF2* (thyroid transcription factor 2) and *TSHR* (thyroid-stimulating hormone receptor) may be associated with thyroid dysplasia and malformations involving the kidneys, lung, forebrain, and palate, in addition to leading to abnormal thyroid follicular cell development [31–33]. Future studies are needed to investigate the full contribution of genetic mutations, copy number variants and environmental factors to CH with ECMs.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2018.11.035.

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