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DUOX2 Mutations Are Frequently Associated With Congenital Hypothyroidism in the Korean Population

Kyoung-Jin Park, M.D.[1,*], Hyun-Kyung Park, M.D.[2,*], Young-Jin Kim, M.D.[2], Kyoung-Ryul Lee, M.D.[2], Jong-Ho Park, B.S.[1], June-Hee Park, B.S.[3], Hyung-Doo Park, M.D.[4], Soo-Youn Lee, M.D.[4], and Jong-Won Kim, M.D.[1,4]

Department of Health Sciences and Technology[1], Samsung Advanced Institute for Health Sciences and Technology, Sungkyunkwan University; Department of Laboratory Medicine[2], Seoul Clinical Laboratories, Seoul Medical Science Institute; Samsung Biomedical Research Institute[3], Samsung Medical Center; Department of Laboratory Medicine & Genetics[4], Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea

Background: Most cases with congenital hypothyroidism (CH) are usually sporadic, while about 20% of the cases are caused by genetic defects. Little information is available regarding the mutation incidence and genetic heterogeneity of CH in Koreans. We aimed to determine the mutation incidence of CH in newborn screenings (NBS) and to evaluate the frequency and spectrum of mutations underlying CH.

Methods: A total of 112 newborns with thyroid dysfunction were enrolled from 256,624 consecutive NBS. Furthermore, 58 outpatients with primary CH were added from an endocrine clinic. All coding exons of *TSHR*, *PAX8*, *TPO*, *DUOX2*, *DUOXA2*, and *SCL5A5* were sequenced.

Results: The mutation incidence of CH was estimated to be 1 in 6,580 newborns. A total of 36 different mutations were identified in 53 cases. The overall mutation positive rate was 31%. The *DUOX2* mutations were the most prevalent in both newborns and outpatients. Seven different recurrent mutations [p.G488R (n=13), p.A649E (n=3), p.R885Q (n=3), p.I1080T (n=2), and p.A1206T (n=2) in *DUOX2*; p.Y138X (n=9) in *DUOXA2*; and p.R450H (n=5) in *TSHR*] were identified as the mutations underlying CH.

Conclusions: The mutation incidence of CH was considerably higher than expected in the Korean newborn population. This study revealed seven different recurrent mutations underlying CH. We conclude that *DUOX2* mutations are a frequent cause of CH in the Korean population.

Key Words: Congenital hypothyroidism, Newborn screening, Incidence, Mutation, *DUOX2*

INTRODUCTION

Congenital hypothyroidism (CH) is one of the most common causes of preventable mental retardation, and it is routinely screened worldwide during the newborn period. Before the introduction of newborn screening (NBS), the incidence of CH was 1 in 6,500 live births, whereas the incidence was initially reported to be approximately 1 in 3,000 newborns with NBS [1]. With more experience from the NBS program, the incidence of CH has been reported to vary considerably among ethnic groups [2-5]. A recent study showed that the overall incidence of CH was 1 in 1,706 in a pan-ethnic group [2].

Compared with the incidence estimated by NBS tests, there are scarce data regarding the incidence of CH associated with mutations in the population. Only a few studies regarding the genetic incidence of CH have been published [6-10]. For instance, the incidence of *TPO* mutations has been reported to be 1 in 66,000 in the Dutch population, 1 in 177,000 in the Japanese population, and 1 in 20,000 in the Slovakian population [6-8]. Further studies in the Japanese populations revealed that

the incidence of *TSHR* mutations, *DUOX2* mutations, and *PAX8* mutations was estimated to be 1 in 118,000, 1 in 44,000, and 1 in 176,000, respectively [9, 10]. These studies suggested that there are ethnic differences in the incidence of CH owing to mutations and that there is genetic heterogeneity underlying CH. Little is known about the genetic incidence of CH in the Korean population. There were only two previous studies regarding genetics of CH in Korean patients with CH [11, 12]. One study in the Korean patients with thyroid dyshormonogenesis (DH) showed a considerably lower rate of mutation positivity in *TPO* mutations [11]. Another study in the Korean patients with CH found that *DUOX2* mutations are a common cause of CH with eutopic thyroid glands [12]. The two previous studies were based on a relatively small series of Korean patients from hospital setting. Until now, little is known on the mutations underlying CH in the Korean population.

Another important consideration is the classification criteria to estimate the incidence of CH. On the basis of the current classification, CH encompasses very distinct morphologic phenotypes, including thyroid dysgenesis (TD) and DH. TD is present in 80-85% of patients, whereas DH accounts for the remaining 15-20% of patients. However, this classification system also includes patients belonging to an intermediate category—those with normal results on thyroid scintigraphy (neither TD nor DH). Exclusion of this intermediate group might influence the variable incidence rate of CH in different populations. The previous genetic epidemiology studies were performed according to a phenotype-first approach (TD or DH) by using this classification [6-10]. Thus, the true genetic incidence of CH remains elusive. To estimate the real incidence of CH associated with mutations, it is necessary to include this intermediate group.

Although TD is usually a sporadic condition, several genes, including *TSHR* and *PAX8*, have been implicated in TD. In contrast, other genes, such as *PDS*, *TPO*, *TG*, *DUOX2*, *DUOXA2*, and *SCL5A5* are involved in thyroid hormone synthesis and secretion (DH). In general, it is expected that DH is inherited in an autosomal recessive manner. However, recent studies have reported notable exceptions and complex genotype-phenotype correlations [12-19]. For instance, biallelic and triallelic mutations in *DUOX2* cause permanent CH, whereas a monoallelic mutation also suffices to cause transient CH [14, 20]. In addition, environmental factors such as iodine excess could delay the onset of CH with biallelic mutations in *DUOX2* [19]. Occasionally, monoallelic mutations of *TPO*, *PAX8*, or *TSHR* might result in transient CH [7, 17, 18]. Interestingly, another study reported that the coexistence of a monoallelic *TPO* mutation

and heterozygous mutations in *TSHR* in the same individual does not influence the severity of the phenotype [15].

We conducted a population-based study regarding the genetic screening of CH directly detected on NBS. This study aimed to determine the incidence of CH associated with mutations in the Korean newborn population. In addition, we evaluated the mutation frequency and spectrum underlying CH and investigated whether there was a genotype-phenotype correlation using the data from outpatients.

METHODS

1. Subjects
We selected subjects from two different situations to evaluate the genetic aspects of CH. In the first situation, genetic screening was conducted in all the newborns with thyroid dysfunction detected on current NBS. We chose this approach in an attempt to include the intermediate group of CH (neither TD nor DH). A total of 112 newborns with thyroid dysfunction were enrolled from 256,624 consecutive NBS at the Seoul Medical Science Institute in Korea from January 2011 to May 2014. The newborn population in this study represented approximately 18% of births (256,624/1,428,400) in Korea during the designated time.

The level of thyroid-stimulating hormone (TSH) was measured in dried blood spots (DBS) by using an AutoDELFIA Neonatal hTSH kit (PerkinElmer, Waltham, MA, USA) on postnatal days 3-5. A total of 1,518 newborns with TSH levels higher than 10 mU/L on the primary screening tests were recalled, and 1,190 of those newborns were retested by using second samples. Thyroid dysfunction was defined as a positive NBS with abnormal TSH levels in the two separate samples. A total of 200 newborns were found to have thyroid dysfunction under these criteria, and ultimately, 112 newborns had a DBS with an adequate amount of DNA to extract for our study. The researchers were blinded to all information regarding the identification of the newborns.

In the second situation, genetic screening was performed in 58 outpatients with confirmed primary CH. They were followed up owing to their primary CH from 2009 to 2014 in an endocrine clinic in the Samsung Medical Center in Korea. For the outpatients, we collected the following information: demographic data, prematurity history, associated anomalies, and the results of the Tc-99m thyroid scan and thyroid ultrasound. This study was approved by the Institutional Review Boards at the Seoul Medical Science Institute and Samsung Medical Center; informed consent from enrolled patients was exempt for this ret-

rospective study that use stored biospecimens.

2. Genetic screening

All DBS were stored at -20°C until the extraction of genomic DNA. Several 3-mm discs from residual DBS were collected by using a Delfia DBS puncher (PerkinElmer). The discs were incubated overnight at 37°C in nuclei lysis buffer (500 μL) and proteinase K (15 μL). After protein precipitation, the tube was centrifuged at 11,400 g for 10 min. The supernatant was transferred to a new tube, and isopropanol (500 μL) was added. After centrifugation at 11,400 g for 2 min, the supernatant was discarded, and 70% ethanol (500 μL) was added. After an additional centrifugation at 11,400 g for 2 min, the pellet was air-dried for 15 min. The concentration of extracted DNA was measured by using the Pico-Green dsDNA assay (Quanti-iTTMPico-Green dsDNA kit; Invitrogen, Carlsbad, CA, USA).

A customized multiplex PCR was applied for 97 genes related to inherited metabolic diseases using the Ion AmpliSeq designer software (Life Technologies, Carlsbad, CA, USA) (manuscript in preparation). Among them, six genes, including genes associated with TD (*TSHR* and *PAX8*) and DH (*TPO*, *DUOX2*, *DUOXA2*, and *SLC5A5*), were selected for genetic screening of CH. All coding exons, intron sequences, and untranslated regions (UTR) of 20-bp flanking each exon were targeted and designed. Regions not covered by the primers among the six genes relevant to CH were 400 bp long, and there were no known mutations found in the Human Genome Mutation Database (HGMD, professional version 2014.01). Targeted sequencing was performed on an Ion PGM sequencer or Ion Proton sequencer (Life Technologies), following the manufacturer's instructions. Sixteen or 20 barcoded samples were sequenced on an Ion 318 chip, and 32 barcoded samples were sequenced on a Proton 1 chip. A total of 170 samples from 112 newborns and 58 outpatients were sequenced.

3. Bioinformatics analysis and mutation prioritization

Data from targeted sequencing were analyzed by using Torrent Suite software (version 4.0.3; Life Technologies). The read was aligned to hg19, and variant calling was performed by using the "Germ Line-PGM-High Stringency" setting. Called variants were functionally annotated by using the ANNOVAR tool [21, 22].

To select the mutations, we applied the following criteria: variants with minor allele frequency (MAF) <0.01 in the 1000 Genome Project (http://browser.1000genomes.org/index.html), the Exome Sequencing Project (http://evs.gs.washington.edu/EVS/), the Exome Aggregation Consortium (http://exac.broadinstitute.

org/), and the Korean Reference Genome Database (http://152.99.75.168/KRGDB/); variants predicted to be "deleterious" or "damaging" from Sorting Intolerant From Tolerant (SIFT), Polymorphism Phenotyping v2 (Polyphen-2), likelihood ratio test (LRT), MutationTaster, MutationAssesor, or FATHMM and with a Genomic Evolutionary Rate Profiling (GERP) score higher than 2; removal of variants located in 3′-UTR, 5′-UTR, downstream, upstream, and noncoding RNAs; and the removal of synonymous variants and selection of missense and nonsense mutations in coding sequences, and splicing mutations. Lastly, the HGMD (professional version 2014.01) was searched to evaluate whether the mutations were novel or known.

4. Sanger sequencing

All prioritized mutations were amplified by PCR using primer pairs designed with Primer3 software. Relevant exons and their flanking intron regions were sequenced by using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) and an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). In case of novel mutations, targeted Sanger sequencing was performed by using 60 healthy individuals as controls.

5. Statistical analyses

The Mann-Whitney U test and Kruskal–Wallis test were used to evaluate the association between TSH level and mutations. The allelic status of the mutations between the newborns and patients was compared with Fisher's exact test. The statistical significance was analyzed with MedCalc version 11.5.1.0 (Mariakerke, Belgium). *P* values less than 0.05 were considered statistically significant.

RESULTS

1. Statistical data of targeted sequencing

The numbers of mapped reads for Ion PGM and Ion Proton were 212,552 and 1,229,961, respectively. The percentage of on-target sequences in each sample showed a median of 90-95%. The median depth of coverage was 92-fold for Ion PGM and 383-fold for Ion Proton, and 99% and 93% of bases showed at least 1-fold and 20-fold coverage, respectively. A total of 1,204 variants were called in 112 newborns. In *TSHR*, *PAX8*, *TPO*, *DUOX2*, *DUOXA2*, and *SCL5A5*, 138 variants were selected. After functional filtering, 47 mutations were prioritized. In the 58 outpatient cases, 28 mutations were prioritized among 1,272 called variants.

Table 1. Mutation incidence and frequency

Genes	Newborn population*				Outpatients[†]	
	Mutation incidence (N=256,624)		Mutation frequency (N=112)		Mutation frequency (N=58)	
	All mutations	Biallelic mutations	All mutations	Biallelic mutations	All mutations	Biallelic mutations
TSHR	1:36,661	1:128,312	7[‡]/112 (6%)	2/112 (2%)	1/58 (2%)	0/58 (0%)
PAX8	1:256,624	ND	1/112 (1%)	0/112 (0%)	2/58 (3%)	0/58 (0%)
TPO	1:85,541	ND	3/112 (3%)	0/112 (0%)	0/58 (0%)	0/58 (0%)
DUOX2	1:13,507	1:85,541	19[‡]/112 (17%)	3/112 (3%)	9/58 (16%)	5/58 (9%)
DUOXA2	1:25,662	1:256,624	10/112 (9%)	1/112 (1%)	2/58 (3%)	1/58 (2%)
SLC5A5	ND	ND	0/112 (0%)	0/112 (0%)	0/58 (0%)	0/58 (0%)
Total	1:6,580	1:42,771	39/112 (35%)	6/112 (5%)	14/58 (24%)	6/58 (10%)

*The newborn population represented approximately 18% of births (256,624/1,428,400) in Korea from January 2011 to May 2014. The mutation incidence and frequency are estimated figures, because sequencing was not performed for all 256,624 subjects; [†]Outpatients with primary congenital hypothyroidism followed up and medicated in the endocrine clinic; [‡]There was one case with concurrent heterozygous mutations in both *TSHR* and *DUOX2*.
Abbreviation: ND, not determined.

2. Incidence

We estimated the incidence of CH based on the current NBS tests to be 1 in 1,283 in the Korean population. The mutation incidence of CH was estimated to be 1 in 6,580 (39/256,624) in the Korean newborn population, according to the mutation status of the six selected genes (Table 1). When limited to biallelic mutations, the mutation incidence of CH was estimated to be 1 in 42,771 (Table 1).

3. Frequency and spectrum of pathogenic variants

Thirty-six different mutations were validated in 53 cases (31%, 53/170), which included 35% of the newborn group (39/112) and 24% of the outpatient group (14/58) (Table 1). Biallelic mutations and monoallelic mutations were identified in 7% (12/170) and 24% (41/170) of the cases, respectively (Table 1). There was a trend that biallelic inactivation was more frequently observed in outpatients than in newborns (10% vs. 5%), although the association was not significant (P=0.059). The mutations consisted of 18 novel mutations and 18 known mutations (Table 2). All of the novel mutations were predicted to have highly deleterious or damaging effects based on the results of several *in silico* analyses (Table 3). On the basis of control databases, such as the 1000 Genome Project, the Exome Sequencing Project, the Exome Aggregation Consortium, and the Korean Reference Genome Database, all of the novel mutant alleles were found to be very rare (Table 3). The relative proportions of mutated genes between the newborns and outpatients were considerably similar; inactivation mutations in *DUOX2* were the most common in both groups (19/112 in newborns and 9/58 in

outpatients, Table 2 and Table 3).

Seven different mutations [p.G488R (n=13), p.A649E (n=3), p.R885Q (n=3), p.I1080T (n=2), and p.A1206T (n=2) in *DUOX2*; p.Y138X (n=9) in *DUOXA2*; and p.R450H (n=5) in *TSHR*) were recurrently observed as the mutations underlying CH. In addition, none of the mutations in *SLC5A5* was found in our study population.

4. Genotype–phenotype relationship study

The relationship between TSH level and mutations showed no significant association under the following criteria: 1) allelic status (biallelic vs. monoallelic inactivation; P=0.1050), 2) classification of CH (genes associated with TD vs. genes associated with DH; P=0.2468), 3) mutated gene (*TSHR* vs. *PAX8* vs. *TPO* vs. *DUOX2* vs. *DUOXA2*; P=0.2582), and 4) study population (screening setting vs. clinic setting; P=0.3838).

Among the 15 outpatients with mutations, four patients were preterm babies (Table 4). Most of the patients with biallelic mutations in *DUOX2* (patient 1 [P1], P2, P4, P6, and P8) had permanent CH. Among them, manifestation of a goiter was found in only one patient (P1). The P4 with triallelic mutations (known heterozygous p.F525S in *TSHR* and biallelic mutations in *DUOX2*) showed no remarkable findings except permanent CH and scoliosis. The other two patients (P3 and P5) with monoallelic mutations in *PAX8* had permanent CH; 1 (P3 with p.S59R) with a history of cryptorchidism and hydrocele showed a mild diffuse enlargement of the thyroid gland, whereas the other (P5 with p.R31C) showed a marked decrease in the uptake of iodide on the thyroid scan. The P11 with a heterozygous mutation

Table 2. Spectrum of mutations identified in this study

Subjects*	TSH level (/T4)	Genes†	NT alteration	AA alteration	Status‡	Results	Subjects*	TSH level (/T4)	Genes†	NT alteration	AA alteration	Status‡	Results
N1	15.8	*DUOXA2*	**c.413dupA**	**p.Y138X**	**Known**	Het	N31	23.3	*DUOXA2*	**c.413dupA**	**p.Y138X**	**Known**	Het
N2	14.2	*DUOX2*	**c.1462G>A**	**p.G488R**	**Known**	Het	N32	14.2	*TSHR*	c.1798T>C	p.C600R	Known	Het
N3	11.9	*DUOX2*	c.4405G>A	p.E1469K	Novel	ComHet	N33	29.5	*PAX8*	c.864+1G>T	NA	Novel	Het
		DUOX2	**c.1462G>A**	**p.G488R**	**Known**		N34	39.1	*TSHR*	c.733G>A	p.G245S	Known	Het
N4	11.8	*TSHR*	**c.1349G>A**	**p.R450H**	**Known**	Hom	N35	127	*DUOX2*	**c.1946C>A**	**p.A649E**	**Known**	ComHet
N5	18.7	*DUOXA2*	**c.413dupA**	**p.Y138X**	**Known**	Het			*DUOX2*	**c.1462G>A**	**p.G488R**	**Known**	
N6	81.6	*DUOX2*	**c.1462G>A**	**p.G488R**	**Known**	Het	N36	14.3	*DUOX2*	c.3179C>T	p.A1060V	Novel	Het
N7	14.9	*DUOX2*	**c.1946C>A**	**p.A649E**	**Known**	Het	N37	14.4	*DUOX2*	c.1219G>T	p.V407F	Novel	Het
N8	12.1	*TPO*	c.1810C>T	p.R604W	Novel	Het	N38	11.2	*TSHR*	**c.1349G>A**	**p.R450H**	**Known**	Het
N9	56.2	*DUOX2*	c.2635G>A	p.E879K	Known	Het	N39	11.4	*TSHR*	**c.1349G>A**	**p.R450H**	**Known**	CoHet
N10	29.2	*TPO*	c.2533G>A	p.G845R	Novel	Het			*DUOX2*	c.3383G>A	p.R1128H	Novel	CoHet
N11	14.7	*DUOX2*	**c.2654G>T**	**p.R885L**	**Known**	Het	P1	27.6/0.5	*DUOX2*	c.4334T>A	p.V1445E	Novel	ComHet
N12	70.2	*DUOXA2*	**c.413dupA**	**p.Y138X**	**Known**	ComHet			*DUOX2*	c.3329G>A	p.R1110Q	Known	
		DUOXA2	c.341G>C	p.G114A	Novel		P2	21.7/0.89	*DUOX2*	**c.3616G>A**	**p.A1206T**	**Known**	ComHet
N13	130	*DUOXA2*	**c.413dupA**	**p.Y138X**	**Known**	Het			*DUOX2*	c.3478delCTG	p.L1160del	Known	
N14	77	*DUOXA2*	c.280C>T	p.R94C	Novel	Het	P3	25.1	*PAX8*	c.177C>G	p.S59R	Novel	Het
N15	19.5	*DUOX2*	**c.1462G>A**	**p.G488R**	**Known**	Het	P4	21.2	*DUOX2*	c.4348T>C	p.Y1450H	Novel	ComHet
N16	10.1	*DUOX2*	**c.3616G>A**	**p.A1206T**	**Known**	Het			*DUOX2*	**c.1462G>A**	**p.G488R**	**Known**	
N17	167	*DUOXA2*	c.535T>C	p.Y179H	Novel	Het			*TSHR*	c.1574T>C	p.F525S	Known	Het
N18	10.8	*DUOX2*	**c.2654G>A**	**p.R885Q**	**Known**	ComHet	P5	23.6	*PAX8*	c.91C>T	p.R31C	Known	Het
		DUOX2	c.2000delT	p.L667fs	Novel		P6	1.3	*DUOX2*	**c.1462G>A**	**p.G488R**	**Known**	Het
N19	36	*DUOXA2*	**c.413dupA**	**p.Y138X**	**Known**	Het	P7	1.64	*DUOX2*	c.4171C>G	p.P1391A	Novel	ComHet
N20	11.2	*DUOXA2*	c.738C>G	p.Y246X	Known	Het			*DUOX2*	**c.1946C>A**	**p.A649E**	**Known**	
N21	200	*TPO*	c.971T>C	p.L324P	Novel	Het	P8	11.5	*DUOX2*	c.2335G>A	p.V779M	Known	ComHet
N22	118	*DUOXA2*	**c.413dupA**	**p.Y138X**	**Known**	Het			*DUOX2*	**c.1462G>A**	**p.G488R**	**Known**	
N23		*DUOX2*	**c.3239T>C**	**p.I1080T**	**Known**	Het	P9	0.03/0.89	*DUOX2*	**c.1462G>A**	**p.G488R**	**Known**	Het
N24	90.1	*DUOX2*	**c.2654G>A**	**p.R885Q**	**Known**	Het	P10	23	*DUOX2*	**c.1462G>A**	**p.G488R**	**Known**	Het
N25	17.8	*DUOX2*	c.1883delA	p.K628fs	Known	Het	P11	19	*TSHR*	c.1349G>A	p.R450H	Known	Het
N26	41.6	*DUOX2*	**c.2654G>A**	**p.R885Q**	**Known**	Het	P12	1.11/0.52	*DUOX2*	**c.3239T>C**	**p.I1080T**	**Known**	Het
N27	13	*TSHR*	**c.1349G>A**	**p.R450H**	**Known**	Het	P13	6.93/0.69	*DUOXA2*	**c.413dupA**	**p.Y138X**	**Known**	Het
N28	12	*DUOX2*	**c.1462G>A**	**p.G488R**	**Known**	Het	P14	12.2	*DUOXA2*	c.148C>T	p.R50C	Novel	ComHet
N29	10.3	*TSHR*	c.614T>C	p.V205A	Novel	ComHet			*DUOXA2*	**c.413dupA**	**p.Y138X**	**Known**	
		TSHR	c.1555C>T	p.R519C	Known		P15	19.5/0.84	*DUOX2*	**c.1462G>A**	**p.G488R**	**Known**	Het
N30	16.4	*DUOX2*	**c.1462G>A**	**p.G488R**	**Known**	Het							

The cutoff levels for thyroid stimulation hormone and free T4 were 10 mU/L and 0.8 ng/dL, respectively. Recurrent mutations are in bold.
*The subjects consisted of a newborn population (N1-N39) and outpatients (P1-P15); †Reference sequences of *TSHR*, *PAX8*, *DUOXA2*, *DUOX2*, and *TPO* were NM_000369, NM_003466, NM_207581, NM_014080, and NM_175721, respectively; ‡Status was evaluated on the basis of the Human Genome Mutation Database (professional version 2014.01), and none of the novel mutations were found in the 60 control individuals.
Abbreviations: TSH, thyroid-stimulating hormone (thyrotropin); T4, thyroxine; NT, nucleotide; AA, amino acid; Het, heterozygote; ComHet, compound heterozygote; Hom, homozygote; NA, not applicable.

Table 3. *In silico* analysis of novel mutations

Gene	AA change	1000G*	ESP†	ExAC‡	KRG§	SIFT	Polyphen2	LRT	Mutation Taster	Mutation Assessor	FATHMM	GERP
TSHR	p.V205A	NA	NA	NA	NA	D	D	D	D	M	D	6.02
PAX8	c.864+1G>T	NA	NA	NA	NA	-	-	-	D	-	-	4.29
	p.S59R	NA	NA	NA	NA	D	D	D	D	H	D	5.31
TPO	p.R604W	NA	NA	0.00002	NA	D	D	N	N	L	T	4.24
	p.G845R	NA	NA	0.00004	NA	T	D	-	N	N	T	-
	p.L324P	NA	NA	NA	NA	D	D	D	D	H	T	4.99
DUOX2	p.E1469K	NA	0.0001	0.00006	NA	D	D	D	D	M	-	5.68
	p.L667fs	NA	NA	NA	NA	-	-	-	-	-	-	-
	p.A1060V	0.0009	NA	0.00016	NA	T	B	D	D	M	-	5.45
	p.V407F	NA	NA	NA	NA	D	P	D	D	M	-	2.14
	p.R1128H	NA	NA	0.00014	NA	D	D	D	D	M	-	5.58
	p.V1445E	NA	NA	NA	NA	D	D	D	D	M	-	4.4
	p.Y1450H	NA	NA	0.00002	NA	D	D	D	D	L	-	5.54
	p.P1391A	NA	NA	0.00001	NA	D	D	D	D	H	-	5.84
DUOXA2	p.G114A	NA	NA	NA	NA	-	D	D	D	L	T	4.66
	p.Y179H	NA	NA	0.00007	0.0016¶	D	D	D	D	M	T	5.3
	p.R94C	NA	NA	NA	NA	D	D	N	N	M	T	5.38
	p.R50C	NA	NA	NA	NA	D	D	D	D	M	T	5.77

Status was evaluated on the basis of the Human Genome Mutation Database (professional version 2014.01). All of the novel variants were very rare based on the 1000 Genome Project, the Exome Sequencing Project, the Exome Aggregation Consortium, and the Korean Reference Genome database. None of the novel variants were found in the 60 control individuals.

*Allele frequency from 1000 Genome Project; †Allele frequency from the Exome Sequencing Project; ‡The Exome Aggregation Consortium; §Korean Reference Genome Database; ¶KNIH1545408908.

Abbreviations: NT, nucleotide; AA, amino acid; GERP, Genomic Evolutionary Rate Profiling; NA, not applicable; D, deleterious or damaging; M, medium; N, neutral; L, low; T, tolerated; B, benign; H, high; P, possibly damaging.

(p.R450H) in *TSHR* showed a marked decrease in the uptake of iodide on the thyroid scan (Table 4).

DISCUSSION

The current diagnostic tests for CH are based on morphological phenotype according to biochemical thyroid function tests, ultrasound, and thyroid scintigraphy. Although these tests can help pinpoint the underlying etiology, they generally provide incomplete information owing to phenotypic heterogeneity (the presence of the intermediate group, with neither TD nor DH). Knowing the exact underlying cause is not necessary during the newborn period because treatment starts without the etiology. However, a genetic diagnosis for CH has added benefits compared with the current phenotype-based approach. First, genetic screening for CH could allow the identification of mutation carriers at risk of recurrent subclinical hypothyroidism after the newborn period. Second, genetic screening might provide a

definite etiologic diagnosis, thus providing insight into the molecular pathogenesis of CH. The genetic diagnosis might also be helpful in case where environmental factors may modify the phenotype of CH, leading to false-negative results.

Thyroid dysfunction detected on NBS is highly heterogeneous; it results from a variety of causes, such as true CH, hyperthyrotropinemia, and subclinical hypothyroidism. A recent study revealed that approximately 30% of newborns with false-positive results in NBS developed persistent subclinical hypothyroidism in their late childhood periods, probably due to genetic abnormalities [23]. Here, we conducted genetic screening for six genes in cases with thyroid dysfunction detected on NBS. This study revealed that there were genetic abnormalities underlying CH in 35% (39/112) of the newborns and in 24% (14/58) of the outpatients. The proportion of mutations was considerably higher than expected in the Korean newborn population; it is known that gene mutations are found in only 2% of cases with TD, which account for 85% of CH [24]. This study

Table 4. Heterogeneous genotype-phenotype correlation in the Korean patients with congenital hypothyroidism

Subjects	Age	Sex	Prematurity	Morphology	Phenotype	Thyroid scan	Associated abnormalities	Zygosity	Gene	NT change	AA change	Status
P1	12 yr	M	Full term	Goiter	Permanent	Mild diffuse enlargement	None	ComHet	*DUOX2*	c.4334T > A c.3329G > A	p.V1445E p.R1110Q	Known Known
P2	12 yr	F	Full term	Normal	Permanent	Normal	Precocious Puberty	ComHet	*DUOX2*	c.3616G > A c.3478delCTG	p.A1206T p.L1160del	Known Known
P3	11 yr	M	Full term	Goiter	Permanent	Mild diffuse enlargement	None	Het	*PAX8*	c.177C > G	p.S59R	Novel
P4	11 yr	M	Full term	Normal	Permanent	Normal	Scoliosis	ComHet Het	*DUOX2* *TSHR*	c.4348T > C **c.1462G > A** c.1574T > C	p.Y1450H **p.G488R** p.F525S	Novel **Known** Known
P5	7 yr	M	Full term	Normal	Permanent	Severely decreased uptake	Hydrocele and cryptorchidism	Het	*PAX8*	c.91C > T	p.R31C	Known
P6	5 yr	F	Premature	Normal	Permanent	NA	Short stature	ComHet	*DUOX2*	c.4171C > G c.1946C > A	p.P1391A p.A649E	Novel Known
P7	6 yr	M	Premature	Normal	Permanent	NA	None	Het	*DUOX2*	**c.1462G > A**	**p.G488R**	**Known**
P8	5 yr	M	Full term	Normal	Permanent	Normal	None	ComHet	*DUOX2*	c.2335G > A **c.1462G > A**	p.V779M **p.G488R**	Known **Known**
P9	3 yr	F	Full term	Normal	Permanent	Normal	None	Het	*DUOX2*	**c.1462G > A**	**p.G488R**	**Known**
P10	3 yr	F	Full term	Normal	Permanent	NA	None	Het	*DUOX2*	**c.1462G > A**	**p.G488R**	**Known**
P11	3 yr	F	Full term	Normal	Permanent	Decreased radioiodine uptake	None	Het	*TSHR*	c.1349G > A	p.R450H	Known
P12	25 months	M	Premature	Normal	ND	NA	PDA	Het	*DUOX2*	c.3239T > C	p.I1080T	Known
P13	20 months	F	Premature	Normal	ND	NA	RDS	Het	*DUOXA2*	**c.413dupA**	**p.Y138X**	**Known**
P14	8 months	F	Premature	Normal	ND	NA	None	ComHet	*DUOXA2*	c.148C > T **c.413dupA**	p.R50C **p.Y138X**	Novel **Known**
P15	6 months	F	Premature	Normal	ND	NA	None	Het	*DUOX2*	**c.1462G > A**	**p.G488R**	**Known**

Status was evaluated on the basis of the Human Genome Mutation Database (professional version 2014.01). Reference sequences of *TSHR*, *PAX8*, *DUOXA2*, and *DUOX2* were NM_000369, NM_003466, NM_207581, and NM_014080, respectively. Recurrent mutations are in bold.
Abbreviations: NT, nucleotide; AA, amino acid; M, male; F, female; NA, not available; ND, not yet determined; PDA, patent ductus arteriosus; RDS, respiratory distress syndrome; Het, heterozygote; ComHet, compound heterozygote; Hom, homozygote.

points to an underestimation of the genetic component in the previous studies, in which genetic screening was conducted without consideration of the intermediate phenotypes [11, 25]. We speculate that there might be yet undetermined genetic components of thyroid dysfunction in NBS.

The genetic incidence based on the mutations of six genes was estimated to be 1 in 6,580 in the Korean newborns. *DUOX2* mutations were a frequent cause of CH associated with mutations in both the NBS setting (17%, 19/112) and outpatient setting (16%, 9/58). A recent study regarding Korean patients reported that *DUOX2* mutations are a common cause of CH (35%, 15/43) [12]. It should be noted that the inclusion criteria for outpatients in our study (primary CH) differed from that in

the previous study (CH with eutopic thyroid glands), thus explaining the higher proportion of *DUOX2* mutations in the previous study [12]. Inone Chinese study, biallelic mutations in *DUOX2* have been identified in approximately 15% (10/67) of patients with CH and goiters [14]. Another Japanese study reported biallelic mutations in *DUOX2* in 28% (4/14) of the patients with permanent DH and 4% (4/102) of the patients with permanent CH. In the present study, biallelic mutations in *DUOX2* were identified in 9% of the patients with primary CH. Considering that most of our outpatients had permanent CH, the contribution of *DUOX2* mutations in the Korean population were higher than that in the Japanese. In contrast, *TPO* mutations were not a major cause of CH in our study, although they

have been reported as the most prevalent cause of DH in Caucasians [8].

In the current study, there was a trend that biallelic inactivation was more frequently observed in outpatients than in newborns, even though there was no statistical significance ($P = 0.059$). The mutation spectrum between them was quite similar, as recurrent mutations in *DUOX2*, *DUOXA2*, and *TSHR* were observed in both newborns and outpatients. Furthermore, we revealed that the genetic screening of outpatients (14/58, 24%) might lead to an underestimation of mutations compared with the genetic screening of newborns (39/112, 35%). Considering the lower positive rate of mutations in outpatients, there might be an incomplete penetrance of mutations for clinical manifestations of CH.

As the previous studies noted, phenotypic manifestations in our outpatients were observed in cases with both monoallelic and biallelic mutations [7, 14, 17-19]. One reason for the high proportion of heterozygote mutations might be attributed to false negatives, unidentified mutations in intronic and regulatory regions, allele dropout due to single-nucleotide polymorphisms in PCR primer binding sites, or structural variations, such as large deletions. Apart from the possibility of false negatives, monoallelic mutations could be sufficient for the pathogenesis of CH.

Recently, the possibility of a non-Mendelian mechanism of CH has been raised, and oligogenic inheritance, triallelic mutations, or synergistic heterozygosity have been suggested [15, 20]. In this study, there was one outpatient with permanent CH associated with triallelic mutations. The patient's phenotype owing to the biallelic *DUOX2* mutations was not aggravated by the coexisting monoallelic *TSHR* mutation. Recently, it has been reported that triallelic mutations in *DUOX2* were associated with permanent CH [20]. Genotype-phenotype correlation studies showed that biallelic/monoallelic mutations in *DUOX2* are associated with permanent CH, whereas monoallelic mutation in *TSHR* has been reported in mild CH [7, 14]. However, little is known about the combined effects of the triallelic mutations involving the two genes. Triallelic mutations involving two different genes have also been reported in other diseases such as telomeropathies [26]. The presence of triallelic mutations is consistent with the concept of synergistic heterozygosity, meaning that multiple heterozygotes in metabolic pathway genes could lead to the disease phenotype [27]. Previous studies on CH reported coexistence of *DUOX2/TSHR* or *TPO/TSHR* mutations in the same individuals [12, 15]. The relationship between the coexisting mutant alleles and the phenotypic manifestations was not conclusive in the previous studies [12, 15]. In the present study,

we could not determine the functional and phenotypic effects of triallelic mutations. Although synergistic heterozygosity would be a good explanation, experimental studies and more genotype-phenotype correlation studies are needed to determine whether the phenotype was influenced by the combination of the mutant alleles. Taken together, the genotype-phenotype correlation is highly complex in CH.

One of the important limitations of this study is that we could not perform functional experiments, although comprehensive *in silico* analyses were done. Another limitation is that we could not completely evaluate genotype-phenotype correlations in newborns with thyroid dysfunction. Furthermore, we did not cover other genes for CH such as *TG* or *DEHAL1*.

In summary, despite the noted limitations, we determined the mutation incidence of CH in a routine NBS setting through population-based genetic screening. This study showed that genetic screening that excludes the intermediate group (neither TD nor DH) might lead to an underestimation of mutations. In addition, we concluded that *DUOX2* mutations were a frequent cause of CH in the Korean population as well as in the Korean patients.

Authors' Disclosures of Potential Conflicts of Interest

Acknowledgments

REFERENCES

1. Grosse SD and Van Vliet G. Prevention of intellectual disability through screening for congenital hypothyroidism: how much and at what level? Arch Dis Child 2011;96:374-9 .
2. Feuchtbaum L, Carter J, Dowray S, Currier RJ, Lorey F. Birth prevalence of disorders detectable through newborn screening by race/ethnicity. Genet Med 2012;14:937-45.
3. Harris KB and Pass KA. Increase in congenital hypothyroidism in New York State and in the United States. Mol Genet Metab 2007;91:268-77.
4. Kurinczuk JJ, Bower C, Lewis B, Byrne G. Congenital hypothyroidism in Western Australia 1981-1998. J Paediatr Child Health 2002;38:187-91.
5. Corbetta C, Weber G, Cortinovis F, Calebiro D, Passoni A, Vigone MC, et al. A 7-year experience with low blood TSH cutoff levels for neonatal screening reveals an unsuspected frequency of congenital hypothyroidism (CH). Clin Endocrinol (Oxf) 2009;71:739-45.

6. Bakker B, Bikker H, Vulsma T, de Randamie JS, Wiedijk BM, De Vijlder JJ. Two decades of screening for congenital hypothyroidism in The Netherlands: TPO gene mutations in total iodide organification defects (an update). J Clin Endocrinol Metab 2000;85:3708-12.

7. Narumi S, Muroya K, Abe Y, Yasui M, Asakura Y, Adachi M, et al. TSHR mutations as a cause of congenital hypothyroidism in Japan: a population-based genetic epidemiology study. J Clin Endocrinol Metab 2009; 94:1317-23.

8. Avbelj M, Tahirovic H, Debeljak M, Kusekova M, Toromanovic A, Krzisnik C, et al. High prevalence of thyroid peroxidase gene mutations in patients with thyroid dyshormonogenesis. Eur J Endocrinol 2007;156: 511-9.

9. Narumi S, Muroya K, Asakura Y, Aachi M, Hasegawa T. Molecular basis of thyroid dyshormonogenesis: genetic screening in population-based Japanese patients. J Clin Endocrinol Metab 2011;96:E1838-42.

10. Narumi S, Muroya K, Asakura Y, Adachi M, Hasegawa T. Transcription factor mutations and congenital hypothyroidism: systematic genetic screening of a population-based cohort of Japanese patients. J Clin Endocrinol Metab 2010;95:1981-5.

11. Lee ST, Lee DH, Kim JY, Kwon MJ, Kim JW, Hong YH, et al. Molecular screening of the TSH receptor (TSHR) and thyroid peroxidase (TPO) genes in Korean patients with nonsyndromic congenital hypothyroidism. Clin Endocrinol (Oxf) 2011;75:715-21.

12. Jin HY, Heo SH, Kim YM, Kim GH, Choi JH, Lee BH, et al. High frequency of DUOX2 mutations in transient or permanent congenital hypothyroidism with eutopic thyroid glands. Horm Res Paediatr 2014;82: 252-60.

13. Satoh M, Aso K, Ogikubo S, Ogasawara A, Saji T. Genetic analysis in children with transient thyroid dysfunction or subclinical hypothyroidism detected on neonatal screening. Clin Pediatr Endocrinol 2009;18:95-100.

14. Wang F, Lu K, Yang Z, Zhang S, Lu W, Zhang L, et al. Genotypes and phenotypes of congenital goitre and hypothyroidism caused by mutations in dual oxidase 2 genes. Clin Endocrinol (Oxf) 2014;81:452-7.

15. Sriphrapradang C, Tenenbaum-Rakover Y, Weiss M, Barkoff MS, Admoni O, Kawthar D, et al. The coexistence of a novel inactivating mutant thyrotropin receptor allele with two thyroid peroxidase mutations: a genotype-phenotype correlation. J Clin Endocrinol Metab 2011;96:E1001-6.

16. Lado-Abeal J, Castro-Piedras I, Palos-Paz F, Labarta-Aizpún JI, Albero-Gamboa R. A family with congenital hypothyroidism caused by a combination of loss-of-function mutations in the thyrotropin receptor and adenylate cyclase-stimulating G alpha-protein subunit genes. Thyroid 2011;21:103-9.

17. Narumi S, Araki S, Hori N, Muroya K, Yamamoto Y, Asakura Y, et al. Functional characterization of four novel PAX8 mutations causing congenital hypothyroidism: new evidence for haploinsufficiency as a disease mechanism. Eur J Endocrinol 2012;167:625-32.

18. Fugazzola L, Cerutti N, Mannavola D, Vannucchi G, Fallini C, Persani L, et al. Monoallelic expression of mutant thyroid peroxidase allele causing total iodide organification defect. J Clin Endocrinol Metab 2003;88: 3264-71.

19. Kasahara T, Narumi S, Okasora K, Takaya R, Tamai H, Hasegawa T. Delayed onset congenital hypothyroidism in a patient with DUOX2 mutations and maternal iodine excess. Am J Med Genet A 2013;161A: 214-7.

20. Fu C, Zhang S, Su J, Luo S, Zheng H, Wang J, et al. Mutation screening of DUOX2 in Chinese patients with congenital hypothyroidism. J Endocrinol Invest 2015;38:1219-24.

21. Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Res 2010;38:e164.

22. Liu X, Jian X, Boerwinkle E. dbNSFP v2.0: a database of human nonsynonymous SNVs and their functional predictions and annotations. Hum Mutat 2013;34:E2393-402.

23. Leonardi D, Polizzotti N, Carta A, Gelsomino R, Sava L, Vigneri R, et al. Longitudinal study of thyroid function in children with mild hyperthyrotropinemia at neonatal screening for congenital hypothyroidism. J Clin Endocrinol Metab 2008;93:2679-85.

24. Rastogi MV and LaFranchi SH. Congenital hypothyroidism. Orphanet J Rare Dis 2010;5:17.

25. Stoppa-Vaucher S, Van Vliet G, Deladoëy J. Variation by ethnicity in the prevalence of congenital hypothyroidism due to thyroid dysgenesis. Thyroid 2011;21:13-8.

26. Collopy LC, Walne AJ, Cardoso S, de la Fuente J, Mohamed M, Toriello H, et al. Triallelic and epigenetic-like inheritance in human disorders of telomerase. Blood 2015;126:176-84.

27. Vockley J. Metabolism as a complex genetic trait, a systems biology approach: implications for inborn errors of metabolism and clinical diseases. J Inherit Metab Dis 2008;31:619-29.