学位論文 **(13-09)**

IDENTIFICATION OF NOVEL GENETIC MUTATIONS IN JAPANESE PATIENTS WITH SEVERE CONGENITAL HYPOTHYROIDISM

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Running head: Mutations in congenital hypothyroidism

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（表 2 つ 図 3 つ 別刷希望部数 20 部 学位審査 2013 年）

Abstract

Objective: The prevalence of genetic mutations in congenital hypothyroidism (CH) remains undetermined. The objective of this study was to determine the prevalence of mutations in *DUOX2*, *TSHR*, *TG*, *PAX8*, and *TPO* among severe permanent primary CH.

Methods: Between April 1999 and March 2011, 114,733 newborns were screened for CH in Akita Prefecture, Japan. Among them, 330 were suspected of having CH and were referred to pediatricians. We recruited 40 patients who were referred to our institute. Among them, we identified 9 permanent primary CH patients who were severely affected with an initial TSH ≥20 mU/l upon newborn screening and performed direct sequencing of the 5 candidate genes.

Results: 3 of 9 patients (33%) had mutations in *PAX8*, *TPO*, and *TSHR*. Among the severely affected subjects, 60% had thyroid dysgenesis (TD), while for patients with initial TSH upon screening <20 mU/l only 12% had TD.

Conclusions: Despite the high frequency of TD, the detection rate of mutations among severe permanent primary CH was higher than expected. This study suggests that the genetic analysis of 5 genes, namely, *DUOX2*, *TSHR*, *TG*, *PAX8*, and *TPO*, is useful for the diagnosis of CH, and that the actual prevalence of

genetic mutations among CH might be higher than as previously estimated.

Key words: congenital hypothyroidism, genes, mutation, prevalence

Introduction

Congenital hypothyroidism (CH) is a highly heterogeneous disorder. CH is classified into permanent and transient CH, which in turn is divided into primary, secondary, or peripheral CH. Most cases of CH are due to primary causes[1]. Thyroid dysgenesis (TD) accounts for 75–85% of permanent primary CH, while thyroid dyshormonogenesis (DH) accounts for 15–20% of cases[2]. TD is generally thought to be a nongenetic disease, but recent evidence points to the possibility of a genetic component[1]. One study reported that 2% of CH patients with TD have a positive familial history[3]. In contrast, DH is generally transmitted in an autosomal recessive manner. Hereditary defects in virtually all of the steps of thyroid hormone biosynthesis and secretion have been described[1].

The prevalence of single genetic mutations in CH remains undetermined. Previous molecular genetic studies have shown that a subset of TD is caused by at least 4 genetic mutations, including *TSHR*[4-6], *PAX8*[7], *NKX2-1*[8], and *FOXE1*[9]. Similarly, at least 7 genes have been implicated in DH, including *TG*[10], *TPO*[11], *SLC5A5*[12], *SLC26A4*[13], *DUOX2*[14], *DUOXA2*[15], and *IYD*[16]. A previous review estimated the prevalence of single genetic mutations in CH to be 5–10%[17].

However, only a few genetic screening studies of CH patients have been conducted[18, 19] and the actual prevalence of most genetic mutations among all CH patients or in the general population has not been investigated.

Recently, Japanese studies have confirmed that more than 20% of permanent primary CH patients have single genetic mutations as determined systematic genetic screening[20-22]. These studies have reported the prevalence of different genetic mutations among 102 CH patients: in descending order of frequency, they occur in *DUOX2*, *TSHR*, *TG*, *PAX8*, and *TPO*.

In this study, we examined the prevalence of genetic mutations in *DUOX2*, *TSHR*, *TG*, *PAX8*, and *TPO* among severe permanent primary CH patients with an initial TSH ≥20 mU/l upon newborn screening in our institute.

Materials and Methods

Subjects

Between April 1999 and March 2011, 114,733 newborns were screened for CH in Akita Prefecture, which is a northern area of Japan (Fig. 1). Among them, 330 with TSH upon screening (whole blood) ≥10 mU/l (April 1999 to July 2004) or ≥9 mU/l (August 2004 to March 2011) were suspected of having CH and were

referred to pediatricians. We recruited 40 patients who were referred to the Department of Pediatrics, Akita University Hospital. Of them, 13 were excluded because they had transient CH, chromosomal abnormalities, and showed movement to other regions. The remaining 27 patients were diagnosed with or suspected of having permanent primary CH and were enrolled in this study. Moreover, the participants were classified into 2 groups according to the severity of their disease: 10 patients had initial TSH upon screening ≥20 mU/l (i.e., referred immediately) and 17 patients had initial TSH upon screening <20 mU/l (i.e., referred after re-examination).

Fig. 1

We selected the 10 severely affected CH patients with initial TSH upon screening ≥20 mU/l for genetic analysis. Written informed consent to participate in the study was obtained from the patients or their parents. This study was approved by the ethical committee of the Akita University Graduate School of Medicine.

Evaluation of patients

During the first visit to our institute, the patients were evaluated for serum levels of TSH, free triiodothyronine (FT3), free thyroxine (FT4), and if possible, thyroglobulin. Thyroid morphology was evaluated by using ultrasonography on

all of the patients. For those patients that were 3–6 years of age, their thyroid function was reevaluated after discontinuation of treatment to distinguish between permanent and transient CH. The patients were evaluated for serum levels of TSH, FT3, FT4, and thyroglobulin. Thyroid morphology was evaluated using both ultrasonography and ^{123}I scintigraphy. The patients who had serum TSH at reevaluation ≥5 mU/l or who had TD were diagnosed with permanent primary CH.

If ^{123}I uptake was increased (≥20%), a perchlorate discharge test was performed. A perchlorate discharge rate >90% indicates the presence of a total iodine organification defect, whereas a rate of 10–90% indicates the presence of a partial iodine organification defect.

Candidate genes

The patients who were diagnosed with or suspected of having permanent primary CH were divided into 3 subgroups: TD (i.e., hypoplasia, aplasia, or ectopia), DH (i.e., iodine organification defect, thyroglobulin synthesis defect, or others), and normal thyroid morphology. *TSHR* and *PAX8* were sequenced for the TD or normal thyroid morphology patients. *TPO* and *DUOX2* were sequenced for DH patients who had an iodine organification defect. *TG* was sequenced for

DH patients who had a thyroglobulin synthesis defect and consequently have typically very low levels of thyroglobulin. Other forms of DH were not included in the study.

Genetic analysis

Genomic DNA was extracted from peripheral blood leukocytes by following a standard technique. Amplification of coding exons and the exon-intron boundary of the candidate genes was performed by using the polymerase chain reaction (PCR) with genomic DNA. The PCR products were purified by using Wizard SV Gel and a PCR Clean-Up System (Promega, Madison, WI, USA). They were then sequenced directly with an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, USA) by using an automated sequencer ABI Prism 310 Genetic Analyzer (Applied Biosystems).

***In silico* analysis of novel genetic mutations**

In order to predict the functional effects of the novel genetic mutations that we identified, we performed *in silico* analysis by using PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/)[23]. This online tool can be used to predict the possible impact of an amino acid substitution on the structure and

function of a human protein. The genetic mutations were classified as being "benign", "possibly damaging", or "probably damaging". In addition to the PolyPhen-2 analysis, we investigated the species conservation among human and other vertebrates of the mutated amino acids by using Evola (http://www.h-invitational.jp/evola/)[24].

Results

Evaluation of patients and genetic analysis

The thyroid morphology of the 2 groups with 27 participants is shown in Table 1. Among the patients with initial TSH upon screening ≥20 mU/l, 6 of 10 patients (60%) had TD. In contrast, among the patients with initial TSH upon screening <20 mU/l, only 2 of 17 patients (12%) had TD.

Table 1

The clinical phenotypes of the patients who had initial TSH upon screening ≥20 mU/l are shown in Table 2. For patient 1–6 who had TD and patient 8 and 9 who had normal thyroid morphology, *TSHR* and *PAX8* were sequenced. For patient 7 who had DH that was indicative of a partial iodine organification defect, *TPO*, and *DUOX2* were sequenced. For patient 10 who had normal thyroid morphology, genetic analysis could not be conducted because she was an

Table 2

infant at the time of enrollment and her thyroid function could not be reevaluated. No patients were suspected to have a thyroglobulin synthesis defect.

Three of 9 patients (33%) had mutations in *PAX8*, *TPO*, and *TSHR* (Table 2). The results of their sequences and family studies of the mutation carriers are shown in Fig. 2 and Fig. 3, respectively. Patient 1 suffered from thyroid hypoplasia and had a novel *PAX8* mutation (heterozygous for p.I34K) (Fig. 2). This missense mutation comprised 2 consecutive point mutations: c.101T > A and c.102C > A. Both of the patient's parents had none of these point mutations (Fig. 3). Patient 7 suffered from a partial iodine organification defect and had a novel *TPO* mutation (compound heterozygous for p.R540X and p.W732L) (Fig. 2). The nonsense mutation for p.R540X was previously reported[25], but the missense mutation for p.W732L was novel. The mother of this patient was heterozygous for p.R540X and the father was heterozygous for p.W732L (Fig. 3). Patient 8 exhibited normal thyroid morphology and had a *TSHR* mutation (homozygous for p.R450H) (Fig. 2), which is common in Japan[26]. The genotypes of the parents were not determined (Fig. 3).

Fig. 2

Fig. 3

***In silico* analysis of novel genetic mutations**

For the PolyPhen-2 analysis, 2 novel mutations (I34K in *PAX8* and W732L in *TPO*) were predicted to be "probably damaging." These mutated amino acids (isoleucine at position 34 of *PAX8* and tryptophan at position 732 of *TPO*) were strictly conserved among 12 other vertebrates.

Discussion

We performed genetic analyses of 5 genes, namely, *DUOX2*, *TSHR*, *TG*, *PAX8*, and *TPO*, for 9 severe permanent primary CH patients with initial TSH ≥20 mU/l upon newborn screening. *DUOX2*, *TG*, and *TPO* were sequenced for the DH patients. *TSHR* and *PAX8* were sequenced for the non-goitrous patients (i.e., TD or normal thyroid morphology) because the thyroid morphologies of these genetic mutations were highly variable: normal, ectopic, hypoplastic, or slightly enlarged[7, 20, 21, 27].

Our study showed that 3 of 9 (33%) subjects had single genetic mutations in *PAX8*, *TPO*, and *TSHR*. Recently, Japanese studies have confirmed by the systematic genetic screening of a population-based cohort of Japanese patients that 23 of 102 (23%) permanent primary CH patients had single genetic mutations[20-22]. These studies identified 8, 6, 5, 2, and 2 mutation carriers in

DUOX2, *TSHR*, *TG*, *PAX8*, and *TPO*, respectively. Although our study subjects were limited to severely affected patients and the sample size was small, our detection rate for mutation carriers was compatible with these studies. Our study suggests that the actual prevalence of single genetic mutations among permanent primary CH patients is higher than was previously estimated[17].

In our study, 6 of 10 patients (60%) with initial TSH ≥20 mU/l upon screening had TD, while only 2 of 17 patients (12%) with initial TSH <20 mU/l had TD. This tendency was also observed in another study. Narumi *et al*. reported that 48 of 70 patients (69%) with serum TSH ≥10 mU/l (under discontinuation of treatment) had TD, while only 3 of 32 patients (9%) with serum TSH <10 mU/l had TD[20]. In addition, recently in Italy the use of a low TSH cutoff for newborn screening revealed that incidence of CH is about two times greater than was previously thought, mainly because of the detection of mild CH within the thyroid gland *in situ*[28]. This fact suggests that TD patients are disposed to high TSH levels upon newborn screening.

Because TD is generally thought to be a non-genetic disease[1], we anticipated a low detection rate for genetic mutations among our severely affected subjects including for the 6 TD patients. In fact, only one of the 6 TD

patients had genetic mutations (*PAX8*). However, the overall detection rate for genetic mutations among our subjects was higher than expected. Our study indicates that severe CH patients may be predisposed to having TD but that genetic analysis may be useful to an extent, especially for *DUOX2*, *TSHR*, *TG*, *PAX8*, and *TPO*.

We identified one *PAX8* mutation carrier among the 6 TD patients. Macchia *et al*. identified 5 *PAX8* mutation carriers among 145 TD patients[7] and Narumi *et al*. identified 2 *PAX8* mutation carriers among 51 TD patients[21]. According to these studies, the prevalence of a *PAX8* mutation is estimated to be 3–4% among TD patients. Notably, almost all of the *PAX8* mutation carriers in our study and the earlier studies had thyroid hypoplasia except for one case that had thyroid ectopia. However, this patient also showed reduced gland size[7]. Indeed, most cases of TD are not hereditary, but *PAX8* mutations can be found rarely in some cases of thyroid hypoplasia.

Among our 9 subjects, we found only one DH patient who had a *TPO* genetic mutation. Mutations in *TPO* appear to be the most common cause of DH with permanent primary CH[1]. Recently, Avbelj *et al*. detected *TPO* mutations in 20 of 43 (46%) permanent primary CH patients with DH in Slovenia, Bosnia, and

Slovakia[29]. In Japan, *DUOX2* mutations have been estimated to be the leading cause of permanent primary CH with DH (1:44,000 newborns)[22], and the estimated Japanese prevalence of *TPO* mutations (1:177,000 newborns)[22] is lower than the Slovene prevalence (1:20,000 newborns)[29] and the Dutch prevalence (1:66,000 newborns)[18]. However, if CH patients show total iodine organification defects or extremely severe thyroid hormone insufficiency, *TPO* then remains the most likely candidate gene for DH.

Japanese studies noted mutations in *DUOX2*, *TG*, or *TPO* in 93% of DH patients (13 of 14) who had at least a goiter (≥2SD), high ^{123}I uptake (≥40%), high perchlorate discharge rate (≥10%), or low saliva-to-plasma ^{123}I ratio (≤10%)[22]. We found only one DH patient among our subjects, but this patient had a goiter (+3.6SD), high ^{123}I uptake (59.4%), and a high perchlorate discharge rate (85%). In fact, we were able to identify a genetic mutation in *TPO*. Although DH accounts for a small proportion of cases of permanent primary CH, the actual prevalence of genetic mutations among DH patients is likely to be fairly high.

We identified one bi-allelic *TSHR* mutation carrier among 8 non-goitrous patients (13%). Our *TSHR* mutation carrier had normal thyroid morphology and

milder phenotypes than our *PAX8* and *TPO* mutation carriers did. The phenotypes of bi-allelic *TSHR* mutations are variable: mild to severe CH and normal to severe hypoplasia[5, 6, 20]. The prevalence of *TSHR* mutations has been presumed to be rare, but a recent Korean study[27] found 13 *TSHR* mutation carriers (16.5%) among 79 patients that were indicative of TD. Similarly, a recent Japanese study[20] found 6 *TSHR* mutation carriers (8%) among 80 non-goitrous patients: 3 patients had bi-allelic mutations with moderate to severe CH and 3 patients had monoallelic mutations with mild CH. The prevalence of *TSHR* mutations could be relatively high among non-goitrous CH patients with phenotypic heterogeneity.

In this study, we did not perform functional analysis of the two novel mutations, *PAX8* I34K and *TPO* W732L. However, there are some reports suggesting the roles of the mutations. Functional analysis of *PAX8* R31H[7] and Q40P[30] showed that these mutations, which are located in the paired domain of *PAX8*, resulted in reduction of the DNA-binding ability of this transcription factor. The *PAX8* I34K mutation is also located in the paired domain, and considered to produce the same effect. Functional analysis of *TPO* R665W and G771R showed that these mutated *TPO* proteins did not migrate to the plasma

membrane[31]. The *TPO* W732L mutation is located between R665W and G771R in the extracellular region[32], and may exhibit the same effect.

A limitation of our study is that the sample size was very small. In Akita Prefecture, the average number of births over the past 13 years has been 8,000–9,000 a year, from which permanent primary CH occurrences are estimated to be 2–3 patients a year. Furthermore, only 12% of the suspected CH patients (40 of 330) were referred to our institute. Another limitation of our study was that genetic mutations were searched for only in the coding region. De Felice *et al*. have pointed out the possibility that mutations in introns or regulatory regions may have been unnoticed and that the prevalence of genetic mutations in TD patients could be underestimated[33]. Nonetheless, we believe that our overall detection rate of genetic mutations among severely affected CH patients is valid, because the characteristics of our subjects and our detection rate of genetic mutations are compatible with those of a recently conducted, population-based Japanese study[20-22].

In conclusion, we identified 2 novel mutations in *PAX8* and *TPO*, and one previously reported mutation in *TSHR* among 9 severe permanent primary CH patients with initial TSH upon screening ≥20 mU/l. In spite of the high

frequency of TD (60%), the overall detection rate of genetic mutations among our subjects was higher than expected. Our study suggests that the genetic analysis of 5 genes, namely, *DUOX2*, *TSHR*, *TG*, *PAX8*, and *TPO*, is useful for the diagnosis of CH, and that the actual prevalence of genetic mutations among CH patients might be higher than was previously estimated. Further studies are required to clarify the roles of the novel mutations, *PAX8* p.I34K and *TPO* p.W732L, in the pathogenesis of primary CH.

Disclosure

None of the authors have any potential conflicts of interest associated with this research.

References

1) Rastogi, M.V. and LaFranchi, S.H. (2010) Congenital hypothyroidism. *Orphanet J. Rare Dis.*, **5**, 17.

2) Grüters, A., Krude, H. and Biebermann, H. (2004) Molecular genetic defects in congenital hypothyroidism. *Eur. J. Endocrinol.*, **151** (Suppl 3), U39–44.

3) Castanet, M., Lyonnet, S., Bonaïti-Pellié, C., Polak, M., Czernichow, P. and Léger, J. (2000) Familial forms of thyroid dysgenesis among infants with congenital hypothyroidism. *N. Engl. J. Med.*, **343**, 441–442.

4) Sunthornthepvarakui, T., Gottschalk, M.E., Hayashi, Y. and Refetoff, S. (1995) Brief report: resistance to thyrotropin caused by mutations in the thyrotropin-receptor gene. *N. Engl. J. Med.*, **332**, 155–160.

5) Biebermann, H., Schöneberg, T., Krude, H., Schultz, G., Gudermann, T. and Grüters, A. (1997) Mutations of the human thyrotropin receptor gene causing thyroid hypoplasia and persistent congenital hypothyroidism. *J. Clin. Endocrinol. Metab.*, **82**, 3471–3480.

6) Abramowicz, M.J., Duprez, L., Parma, J., Vassart, G. and Heinrichs, C. (1997) Familial congenital hypothyroidism due to inactivating mutation

of the thyrotropin receptor causing profound hypoplasia of the thyroid gland. *J. Clin. Invest.*, **99**, 3018–3024.

7) Macchia, P.E., Lapi, P., Krude, H., *et al.* (1998) PAX8 mutations associated with congenital hypothyroidism caused by thyroid dysgenesis. *Nat. Genet.*, **19**, 83–86.

8) Devriendt, K., Vanhole, C., Matthijs, G. and de Zegher, F. (1998) Deletion of thyroid transcription factor-1 gene in an infant with neonatal thyroid dysfunction and respiratory failure. *N. Engl. J. Med.*, **338**, 1317–1318.

9) Clifton-Bligh, R.J., Wentworth, J.M., Heinz, P., Crisp, M.S., John, R., Lazarus, J.H., Ludgate, M. and Chatterjee, V.K. (1998) Mutation of the gene encoding human TTF-2 associated with thyroid agenesis, cleft palate and choanal atresia. *Nat. Genet.*, **19**, 399–401.

10) Ieiri, T., Cochaux, P., Targovnik, H.M., Suzuki, M., Shimoda, S., Perret, J. and Vassart, G. (1991) A 3' splice site mutation in the thyroglobulin gene responsible for congenital goiter with hypothyroidism. *J. Clin. Invest.*, **88**, 1901–1905.

11) Abramowicz, M.J., Targovnik, H.M., Varela, V., Cochaux, P., Krawiec, L.,

Pisarev, M.A., Propato, F.V., Juvenal, G., Chester, H.A. and Vassart, G. (1992) Identification of a mutation in the coding sequence of the human thyroid peroxidase gene causing congenital goiter. *J. Clin. Invest.*, **90**, 1200–1204.

12) Fujiwara, H., Tatsumi, K., Miki, K., Harada, T., Miyai, K., Takai, S. and Amino, N. (1997) Congenital hypothyroidism caused by a mutation in the Na+/I- symporter. *Nat. Genet.*, **16**, 124–125.

13) Everett, L.A., Glaser, B., Beck, J.C., *et al.* (1997) Pendred syndrome is caused by mutations in a putative sulphate transporter gene (PDS). *Nat. Genet.*, **17**, 411–422.

14) Moreno, J.C., Bikker, H., Kempers, M.J., van Trotsenburg, A.S., Baas, F., de Vijlder, J.J., Vulsma, T. and Ris-Stalpers, C. (2002) Inactivating mutations in the gene for thyroid oxidase 2 (THOX2) and congenital hypothyroidism. *N. Engl. J. Med.*, **347**, 95–102.

15) Zamproni, I., Grasberger, H., Cortinovis, F., *et al.* (2008) Biallelic inactivation of the dual oxidase maturation factor 2 (DUOXA2) gene as a novel cause of congenital hypothyroidism. *J. Clin. Endocrinol. Metab.*, **93**, 605–610.

16) Moreno, J.C., Klootwijk, W., van Toor, H., Pinto, G., D'Alessandro, M., Lèger, A., Goudie, D., Polak, M., Grüters, A. and Visser, T.J. (2008) Mutations in the iodotyrosine deiodinase gene and hypothyroidism. *N. Engl. J. Med.*, **358**, 1811–1818.

17) Krude, H., Biebermann, H., Schnabel, D., Ambrugger, P. and Grüters, A. (2000) Molecular pathogenesis of neonatal hypothyroidism. *Horm. Res.*, **53** (Suppl 1), 12–18.

18) Bakker, B., Bikker, H., Vulsma, T., de Randamie, J.S., Wiedijk, B.M. and De Vijlder, J.J. (2000) Two decades of screening for congenital hypothyroidism in The Netherlands: TPO gene mutations in total iodide organification defects (an update). *J. Clin. Endocrinol. Metab.*, **85**, 3708–3712.

19) Hishinuma, A., Fukata, S., Nishiyama, S., *et al.* (2006) Haplotype analysis reveals founder effects of thyroglobulin gene mutations C1058R and C1977S in Japan. *J. Clin. Endocrinol. Metab.*, **91**, 3100–3104.

20) Narumi, S., Muroya, K., Abe, Y., Yasui, M., Asakura, Y., Adachi, M. and Hasegawa, T. (2009) TSHR mutations as a cause of congenital hypothyroidism in Japan: a population-based genetic epidemiology study.

J. Clin. Endocrinol. Metab., **94**, 1317–1323.

21) Narumi, S., Muroya, K., Asakura, Y., Adachi, M. and Hasegawa, T. (2010) Transcription factor mutations and congenital hypothyroidism: systematic genetic screening of a population-based cohort of Japanese patients. *J. Clin. Endocrinol. Metab.*, **95**, 1981–1985.

22) Narumi, S., Muroya, K., Asakura, Y., Adachi, M. and Hasegawa, T. (2011) Molecular basis of thyroid dyshormonogenesis: genetic screening in population-based Japanese patients. *J. Clin. Endocrinol. Metab.*, **96**, E1838–1842.

23) Adzhubei, I.A., Schmidt, S., Peshkin, L., Ramensky, V.E., Gerasimova, A., Bork, P., Kondrashov, A.S. and Sunyaev, S.R. (2010) A method and server for predicting damaging missense mutations. *Nat. Methods*, **7**, 248–249.

24) Matsuya, A., Sakate, R., Kawahara, Y., *et al.* (2008) Evola: Ortholog database of all human genes in H-InvDB with manual curation of phylogenetic trees. *Nucleic Acids Res.*, **36**, D787–792.

25) Bikker, H., Vulsma, T., Baas, F. and de Vijlder, J.J. (1995) Identification of five novel inactivating mutations in the human thyroid peroxidase gene

by denaturing gradient gel electrophoresis. *Hum. Mutat.*, **6**, 9–16.

26) Tsunekawa, K., Onigata, K., Morimura, T., Kasahara, T., Nishiyama, S., Kamoda, T., Mori, M., Morikawa, A. and Murakami, M. (2006) Identification and functional analysis of novel inactivating thyrotropin receptor mutations in patients with thyrotropin resistance. *Thyroid*, **16**, 471–479.

27) Lee, S.T., Lee, D.H., Kim, J.Y., Kwon, M.J., Kim, J.W., Hong, Y.H., Lee, Y.W. and Ki, C.S. (2011) Molecular screening of the TSH receptor (TSHR) and thyroid peroxidase (TPO) genes in Korean patients with nonsyndromic congenital hypothyroidism. *Clin. Endocrinol.*, **75**, 715–721.

28) Corbetta, C., Weber, G., Cortinovis, F., Calebiro, D., Passoni, A., Vigone, M.C., Beck-Peccoz, P., Chiumello, G. and Persani, L. (2009) A 7-year experience with low blood TSH cutoff levels for neonatal screening reveals an unsuspected frequency of congenital hypothyroidism (CH). *Clin. Endocrinol.* (Oxf), **71**, 739–745.

29) Avbelj, M., Tahirovic, H., Debeljak, M., Kusekova, M., Toromanovic, A., Krzisnik, C. and Battelino, T. (2007) High prevalence of thyroid

peroxidase gene mutations in patients with thyroid dyshormonogenesis. *Eur. J. Endocrinol.*, **156**, 511–519.

30) Congdon, T., Nguyen, L.Q., Nogueira, C.R., Habiby, R.L., Medeiros-Neto, G. and Kopp P. (2001) A novel mutation (Q40P) in PAX8 associated with congenital hypothyroidism and thyroid hypoplasia: evidence for phenotypic variability in mother and child. *J. Clin. Endocrinol. Metab.*, **86**, 3962–3967.

31) Umeki, K., Kotani, T., Kawano, J., Suganuma, T., Yamamoto, I., Aratake, Y., Furujo, M. and Ichiba, Y. (2002) Two novel missense mutations in the thyroid peroxidase gene, R665W and G771R, result in a localization defect and cause congenital hypothyroidism. *Eur. J. Endocrinol.*, **146**, 491–498.

32) Libert, F., Ruel, J., Ludgate, M., Swillens, S., Alexander, N., Vassart, G. and Dinsart C. (1987) Thyroperoxidase, an auto-antigen with a mosaic structure made of nuclear and mitochondrial gene modules. *EMBO J.*, **20**, 4193–4196.

33) De Felice, M. and Di Lauro, R. (2004) Thyroid development and its disorders: genetics and molecular mechanisms. *Endocr. Rev.*, **25**, 722–

746.

Figures Legends

Fig. 1. Study subject enrollment. In total, 114,733 newborns were screened for CH in Akita Prefecture between April 1999 and March 2011. Three hundred thirty newborns with TSH upon screening (whole blood) ≥10 mU/l (April 1999 to July 2004) or ≥9 mU/l (August 2004 to March 2011) were referred to pediatricians. Among them, 40 were referred to our institute. Of these patients, 27 were diagnosed with or suspected of having permanent primary CH and were enrolled in this study. Moreover, according to the severity of their disease, the participants were classified into 2 groups: 10 patients with initial TSH upon screening ≥20 mU/l (i.e., referred immediately) and 17 patients with initial TSH upon screening <20 mU/l (i.e., referred after re-examination).

Fig. 2. Three genetic mutations identified in severe permanent primary CH patients. The identified *PAX8* mutation is heterozygous for a novel mutation p.I34K. This missense mutation comprises 2 consecutive point mutations: c.101T > A and c.102C > A. The identified *TPO* mutation is compound heterozygous for p.R540X and p.W732L with the latter being a novel mutation.

The identified *TSHR* mutation is homozygous for p.R450H, which is common in Japan.

Fig. 3. Family studies of the 3 mutation carriers. Both parents of Patient 1 with a *PAX8* mutation had no mutation. Both parents of Patient 7 with a *TPO* mutation were heterozygous for R540X and W732L, respectively. The genotypes of the parents of Patient 8 with a *TSHR* mutation were not determined.

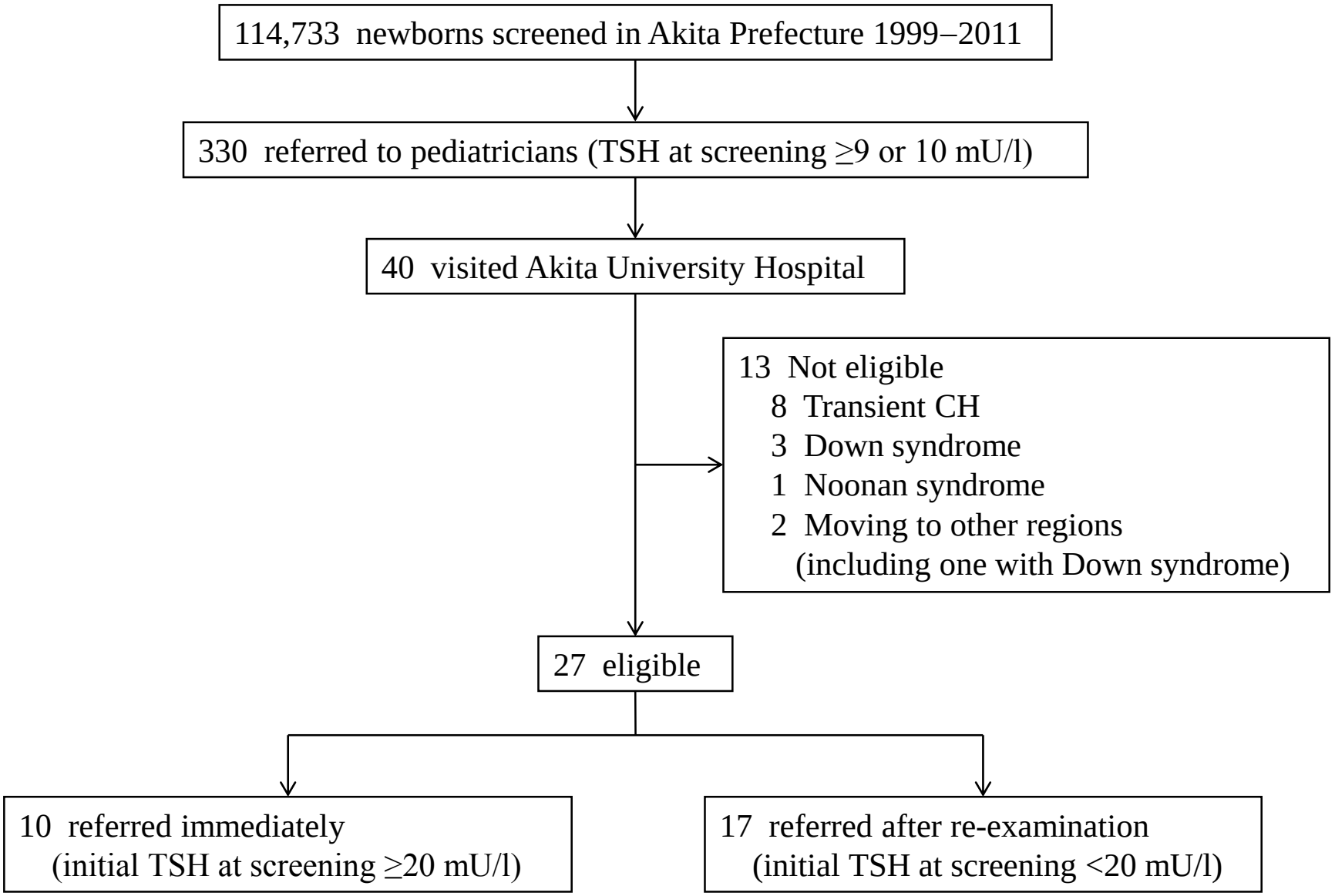
114,733 newborns screened in Akita Prefecture 1999–2011
330 referred to pediatricians (TSH at screening ≥9 or 10 mU/l)
40 visited Akita University Hospital
13 Not eligible
8 Transient CH
3 Down syndrome
1 Noonan syndrome
2 Moving to other regions
(including one with Down syndrome)
27 eligible
10 referred immediately
(initial TSH at screening ≥20 mU/l)
17 referred after re-examination
(initial TSH at screening <20 mU/l)

Fig. 1

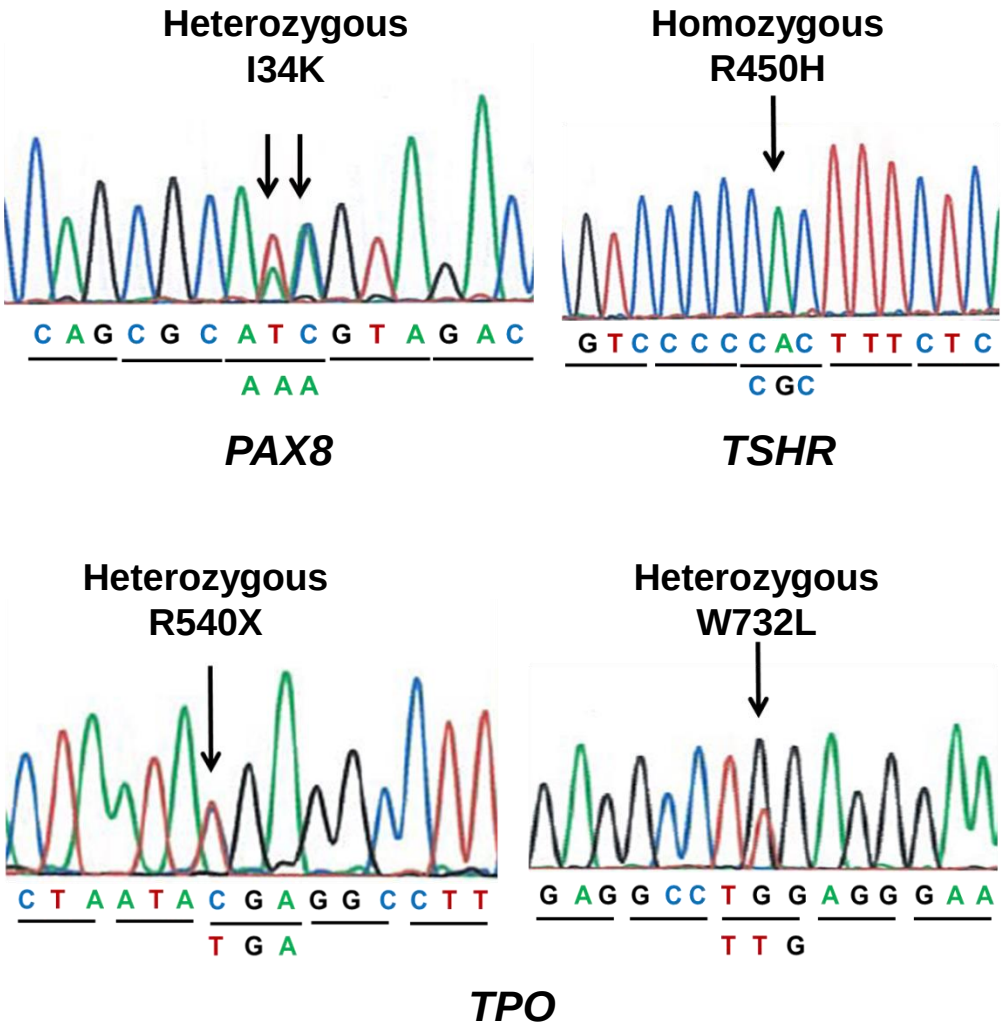
Heterozygous
I34K
C A G C G C A T C G T A G A C
A A A
PAX8
Homozygous
R450H
G T C C C C C A C T T T C T C
C G C
TSHR
Heterozygous
R540X
C T A A T A C G A G G C C T T
T G A
Heterozygous
W732L
G A G G C C T G G A G G G A A
T T G
TPO

Fig. 2

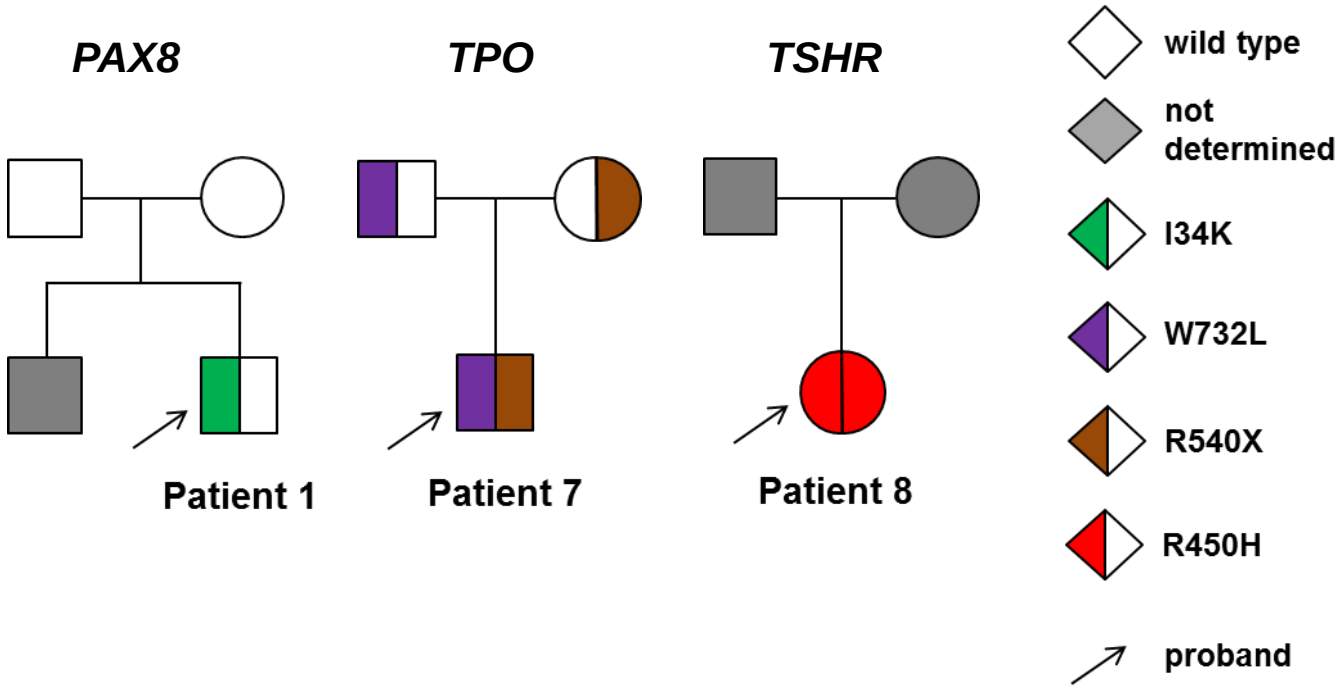
PAX8
TPO
TSHR
wild type
not determined
I34K
W732L
R540X
R450H
proband
Patient 1
Patient 7
Patient 8

Fig. 3

Table 1. Thyroid morphologies of the participants

Variable	Initial TSH at screening ≥20 mU/l	Initial TSH at screening <20 mU/l
n (males/females)	10 (2/8)	17 (6/11)
Thyroid morphology, n		
Normal	3	13
Hypoplasia	3	2
Aplasia	2	0
Ectopia	1	0
Goiter	1	1
Not evaluated	0	1

Table 2. The clinical phenotypes of the patients with initial TSH at screening ≥20 mU/l

Patient No.	Age (yrs), sex	Thyroid morphology	Gene mutation	Initial TSH at screening (mU/l)	Serum TSH at reevaluation (mU/l)	FT4 at reevaluation (ng/dl)	Tg (ng/ml)	^{123}I uptake (3h)	Perchlorate discharge rate (%)
1	9, M	Hypoplasia	*PAX8*	236.7	282.3	<0.1	89.8	4.8	N.A.
2	10, F	Hypoplasia	–	89.5	292.1	<0.4	78.7	5.3	N.A.
3	11, F	Hypoplasia	–	58.4	315.8	N.A.	13.0	N.A.	N.A.
4	8, F	Aplasia	–	>200	184.6	<0.1	<8	8.3	N.A.
5	1, F	Aplasia	–	>93.3	N.A.	N.A.	68.1	N.A.	N.A.
6	10, F	Ectopia	–	95	198.0	0.2	45.1	3.7	N.A.
7	12, M	Goiter	*TPO*	334.4	169.6	0.9	>800	59.4	85
8	6, F	Normal	*TSHR*	25.7	22.8	0.9	24.6	12.9	2.7
9	13, F	Normal	–	>100	226.7	0.1	N.A.	7	N.A.
10	1, F	Normal	Not done	33.1	N.A.	N.A.	124	N.A.	N.A.

FT4, free thyroxine; Tg, thyroglobulin; M, male; F, female; N.A., not available; –, not detectable