periods. Results include the alleviation of symptoms of hyperthyroidism in PRTH (15,16,18,19), suppression of TSH and/or T4 levels (10,13–20), and the normalization of neurological symptoms, including nervousness, inattention, and hyperkinetic behaviors (15,17). TRIAC was shown to reduce the goiter size in non-RTH euthyroid individuals more effectively than L-thyroxine (28). Our report strengthens the above findings by the quantitative demonstration of TRIAC effects: (i) resolution of SVPC, (ii) reduction in the goiter size by almost half as determined by US, and (iii) decreases in both FT4 and T4 levels depending on TRIAC dosage. To our knowledge, TRIAC treatment for 3 years is one of the longest treatments published in the literature. However, to ensure the long-term safety of TRIAC treatment, observation of a larger number of cases is mandatory.

The close association between RTH and ADHD is well known, and the prevalence of ADHD in pediatric RTH has been estimated to be as high as 70%, 10 times higher than that in the general population (29). In such cases, the usual medication prescribed for ADHD in children, methylphenidate, for example, has been shown to be minimally effective. Our patient showed distinct symptoms indicating the presence of ADHD: according to the evaluation of the child psychiatrist, he had hyperactivity, inattention, and impulsiveness, which were inconsistent with his intellectual level. In addition, the presence of autism seemed unlikely. Although the absolute score of 21 in ADHD Rating Scale-IV before therapy was not grossly high, this discrepancy may be derived from the fact that the score was rated by the parents.

TRIAC therapy in our RTH patient resulted in amelioration of ADHD symptoms, as early as 6 months after initiation of treatment, with a decrease in ADHD Rating Scale-IV score (from 21 to 9). This improvement may in part be derived from factors other than TRIAC, including spontaneous improvement or maturity. Nevertheless, the effectiveness of TRIAC in ADHD has been demonstrated previously in pediatric patients, both in GRTH (17) and PRTH (15) cases. The findings in our patient support these previous reports. Although the effect of TRIAC on ADHD can be confirmed only after a placebo-controlled trial, the use of TRIAC to treat RTH children with overt ADHD symptoms seems to be justified at present.

Elevated T3 during TRIAC therapy, which was documented repeatedly (7,10,18,20), has been considered to be due to antibody crossreactivity with serum TRIAC. Through the spiked recovery test, we could demonstrate that the degree of crossreaction differs considerably according to the methods of measuring, probably due to the kind of antibodies used. However, a linear relationship was observed between the TRIAC added to the sample and the apparent T3 increment; this indicates that crossreactivity was constant at various TRIAC levels in each method, and thus T3 levels can be used as a surrogate for serum TRIAC levels. On the other hand, a difference of about 15%–20% in crossreactivity was observed depending on whether the medium was pooled or hypothyroid serum. A clear explanation for this is unknown, but it may indicate that the serum T3 level influences the degree of crossreactivity of TRIAC. Nevertheless, T3 levels were used as a TRIAC surrogate in the pharmacological study, because the study was conducted with constant T3 levels.

In accordance with a classic study on specific radioimmunoassay for TRIAC (30), TRIAC (T3 surrogate) disappeared

TABLE 4. LITERATURE REVIEW OF TRIAC THERAPY FOR THYROID HORMONE RESISTANCE

Case	*Sex*	*Age (y)*	*Maintenance dose*	*Administration*	*Duration*	*Category*	*Mutation*	*Effects*	*Ref.*
1	M	0	1.4 mg	NA	7 months	PRTH	1590_1591insT	TSH↓ PR↓ decrease in sweating	19
2	M	2	1.4 mg	Twice a day	4 months	PRTH	NA	Not effective	7
3	M	3–5	1.4–1.75 mg	Twice a day	24 months	PRTH	P453T	TSH↓ FT4↓ PR↓ improvement in nervousness, hyperkinetic behavior, and heat intolerance	15
4	M	6–7	1.4mg	Twice a day	12 months	GRTH	F455I	TSH↓ FT4↓ improvement in hyperactivity	17
5	F	10	0.7 mg	Twice a day	14 months	PRTH	NA	TSH↓ FT4↓ PR↓ reduction in thyroid volume	18
6	F	11–13	2.1 mg	NA	22 months	PRTH	NA	TSH↓ FT4↓ PR↓ reduction in thyroid volume	16
7	F	15	1.4–2.1 mg	NA	9 weeks	PRTH	NA	TSH↓ reduction in thyroid volume	9
8	F	17	0.7–2.1 mg	NA	4 months	GRTH	H435L	FT4↓ reduction in thyroid volume	31
9	F	36	1.05–2.1 mg	NA	4 months	PRTH	H435Q	TSH↓ FT4↓ PR↓ tremor↓ reduction in thyroid volume	
10	F	37	3.5 mg	Twice a day	28 weeks	PRTH	NA	TSH↓ FT4↓ reduction in thyroid volume	20
11	M	42	2.8–4.2 mg	Twice a day	6 weeks	GRTH	NA	TSH↓ FT4↓	
12	F	47	2.1 mg	NA	4 months	PRTH	NA	TSH↓ FT4↓ TC↑ PR↓	14
13	F	38	1.4 mg	NA	3 months	PRTH	NA	TSH↓	10
14	M	38	1.4 mg	NA	3 months	PRTH	NA	TSH↓	
15	NA	NA	2.1 mg	NA	58 weeks	PRTH	NA	TSH↓ reduction in thyroid volume	13
16	NA	NA	2.1 mg	NA	25 weeks	PRTH	NA	TSH↓ FT4↓ reduction in thyroid volume	

M, male; F, female; PRTH, pituitary thyroid hormone resistance; GRTH, generalized thyroid hormone resistance; ↓, decrease; ↑, increase; PR, pulse rate; TC, total cholesterol; NA, not available

from circulation rapidly in both normal subjects and patients. On the other hand, TRIAC showed a sustained TSH inhibitory effect, which also supports previous reports (30). However, we observed a substantial difference in this inhibitory effect between the healthy volunteer and our RTH patient: inhibition lasted for at least 24 hours in the former, whereas it lasted for <12 hours in the latter. Ueda and colleagues also described a reduced TSH inhibitory effect of TRIAC in RTH patients (31). Although the rationale for this phenomenon is unknown, TRIAC administered twice a day may be recommended in patients with hyperthyroidism due to RTH. Considering the discrepancy between the circulating TRIAC level and its pharmacological effect, TRIAC serum levels are not a suitable guide in deciding treatment dosage. However, it may be used as an indicator of the patient's compliance.

The long treatment period enabled us to evaluate the relationship between TRIAC dose and thyroid function, and we found that the effect of TRIAC on lowering T4 and FT4 seemed dose dependent. Therefore, in a therapeutic regimen, a stepwise increment of the TRIAC dose, guided by both clinical observations and TSH and (F)T4 levels, may be prudent. Table 4 presents a review of the hitherto-reported TRIAC therapy regimes, which may be helpful in TRIAC dose optimization.

In conclusion, TRIAC therapy for 3 years for a boy with PRTH was safe and effective in ameliorating hyperthyroidism as well as ADHD symptoms. Although TRIAC showed a long TSH inhibitory effect despite its short half-life, TRIAC should be given using a divided dose regimen, considering the shorter drug effect in RTH patients. Because TRIAC seems to work dose dependently, its dose should be adjusted according to the clinical effects and laboratory findings.

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Disclosure Statement

The authors declare that they have no commercial associations that might create an actual or potential conflict of interest in connection with the work described in the article.

References

1. Refetoff S, Dumitrescu AM 2007 Syndromes of reduced sensitivity to thyroid hormone: genetic defects in hormone receptors, cell transporters and deiodination. Best Pract Res Clin Endocrinol Metab **2:**277–305.
2. Ercan O 2003 Thyroid Hormone Resistance in children. Pediatr Endocrinol Rev **1(Suppl 2):**191–198.
3. Cheng SY, Leonard JL, Davis PJ 2010 Molecular aspects of thyroid hormone actions. Endocr Rev **31:**139–170.
4. Beck-Peccoz P, Forloni F, Cortelazzi D, Persani L, Papandreou MJ, Asteria C, Faglia G 1992 Pituitary resistance to thyroid hormones. Horm Res **38:**66–72.
5. Beck-Peccoz P, Chatterjee VKK 1994 The variable clinical phenotype in thyroid hormone resistance syndrome. Thyroid **4:**225–232.
6. Blair JC, Mohan U, Larcher VF, Rajanayagam O, Burrin JM, Perry LA, Grossman AB, Chatterjee VK, Savage MO 2002 Neonatal thyrotoxicosis and maternal infertility in thyroid hormone resistance due to a mutation in the TRbeta gene (M313T). Clin Endocrinol (Oxf) **57:**405–409.
7. Hamon P, Bovier-Lapierre M, Robert M, Peynaud D, Pugeat M, Orgiazzi J 1988 Hyperthyroidism due to selective pituitary resistance to thyroid hormones in a 15-month-old boy: efficacy of D-thyroxine therapy. J Clin Endocrinol Metab **67:** 1089–1093.
8. Kim TJ, Travers S 2008 Case report: thyroid hormone resistance and its therapeutic challenges. Curr Opin Pediatr **20:**490–493.
9. Dulgeroff AJ, Geffner ME, Koyal SN, Wong M, Hershman JM 1992 Bromocriptine and Triac therapy for hyperthyroidism due to pituitary resistance to thyroid hormone. J Clin Endocrinol Metab **75:**1071–1075.
10. Salmela PI, Wide L, Juustila H, Ruokonen A 1988 Effects of thyroid hormones (T4,T3), bromocriptine and Triac on inappropriate TSH hypersecretion. Clin Endocrinol (Oxf) **28:**497–507.
11. Beck-Peccoz P, Medri G, Piscitelli G, Mariotti S, Bertoli A, Barbarino A, Rondena M, Martino E, Pinchera A, Faglia G 1988 Treatment of inappropriate secretion of thyrotropin with somatostatin analog SMS 201–995. Horm Res **29:**121–123.
12. Dorey F, Strauch G, Gayno JP 1990 Thyrotoxicosis due to pituitary resistance to thyroid hormones. Successful control with D thyroxine: a study in three patients. Clin Endocrinol (Oxf) **32:**221–228.
13. Faglia G, Beck-Peccoz P, Piscitelli G, Medri G 1987 Inappropriate secretion of thyrotropin by the pituitary. Horm Res **26:**79–99.
14. Beck-Peccoz P, Piscitelli G, Cattaneo MG, Faglia G 1983 Successful treatment of hyperthyroidism due to non-neoplastic pituitary TSH hypersecretion with 3,5,3′-triiodothyroacetic acid (TRIAC). J Endocrinol Invest **6:** 217–223.
15. Radetti G, Persani L, Molinaro G, Mannavola D, Cortelazzi D, Chatterjee VK, Beck-Peccoz P 1997 Clinical and hormonal outcome after two years of triiodothyroacetic acid treatment in a child with thyroid hormone resistance. Thyroid **7:**775–778.
16. Darendeliler F, Baş F 1997 Successful therapy with 3,5,3′-triiodothyroacetic acid (TRIAC) in pituitary resistance to thyroid hormone. J Pediatr Endocrinol Metab **10:**535–538.
17. Torre P, Bertoli M, Di Giovanni S, Scommegna S, Conte C, Novelli G, Cianfarani S 2005 Endocrine and neuropsychological assessment in a child with a novel mutation of thyroid hormone receptor: response to 12-month triiodothyroacetic acid (TRIAC) therapy. J Endocrinol Invest **28:**657–662.
18. Crinò A, Borrelli P, Salvatori R, Cortelazzi D, Roncoroni R, Beck-Peccoz P 1992 Anti-iodothyronine autoantibodies in a girl with hyperthyroidism due to pituitary resistance to thyroid hormones. J Endocrinol Invest **15:**113–120.
19. Wu SY, Cohen RN, Simsek E, Senses DA, Yar NE, Grasberger H, Noel J, Refetoff S, Weiss RE 2006 A novel thyroid hormone receptor-beta mutation that fails to bind nuclear receptor corepressor in a patient as an apparent cause of severe, predominantly pituitary resistance to thyroid hormone. J Clin Endocrinol Metab **91:**1887–1895.

20. Kunitake JM, Hartman N, Henson LC, Lieberman J, Williams DE, Wong M, Hershman JM 1989 3,5,3′-triiodothyroacetic acid therapy for thyroid hormone resistance. J Clin Endocrinol Metab **69:**461–466.
21. Weiss RE 1999 Management patients with resistance to thyroid hormone. Thyroid Today **12:**1–11.
22. DuPaul GJ, Power TJ, Anastopoulos AD, Reid R 1998 ADHD Rating Scale-IV: Checklists, Norms, and Clinical Interpretation. The Guilford Press, New York, NY.
23. Parrilla R, Mixson AJ, McPherson JA, McClaskey JH, Weintraub BD 1991 Characterization of seven novel mutations of the c-erbA beta gene in unrelated kindreds with generalized thyroid hormone resistance. Evidence for two "hot spot" regions of the ligand binding domain. J Clin Invest **88:**2123–2130.
24. Shuto Y, Wakabayashi I, Amuro N, Minami S, Okazaki T 1992 A point mutation in the 3,5,3′-triiodothyronine-binding domain of thyroid hormone receptor-beta associated with a family with generalized resistance to thyroid hormone. J Clin Endocrinol Metab **75:**213–217.
25. Usala SJ, Tennyson GE, Bale AE, Lash RW, Gesundheit N, Wondisford FE, Accili D, Hauser P, Weintraub BD 1990 A base mutation of the C-erbA beta thyroid hormone receptor in a kindred with generalized thyroid hormone resistance. Molecular heterogeneity in two other kindreds. J Clin Invest **85:**93–100.
26. Sato H, Sakai H 2006 A family showing resistance to thyroid hormone associated with chronic thyroiditis and its clinical features: a case report. Endocr J **53:**421–425.
27. von Olshausen K, Bischoff S, Kahaly G, Mohr-Kahaly S, Erbel R, Beyer J, Meyer J 1989 Cardiac arrhythmias and heart rate in hyperthyroidism. Am J Cardiol **63:**930–933.
28. Brenta G, Schnitman M, Fretes O, Facco E, Gurfinkel M, Damilano S, Pacenza N, Blanco A, Gonzalez E, Pisarev MA 2003 Comparative efficacy and side effects of the treatment of euthyroid goiter with levo-thyroxine or triiodothyroacetic acid. J Clin Endocrinol Metab **88:** 5287–5292.
29. Hauser P, Zametkin AJ, Martinez P, Vitiello B, Matochik JA, Mixson AJ, Weintraub BD 1993 Attention deficit-hyperactivity disorder in people with generalized resistance to thyroid hormone. N Engl J Med **328:**997–1001.
30. Menegay C, Juge C, Burger AG 1989 Pharmacokinetics of 3,5,3′-triiodothyroacetic acid and its effects on serum TSH levels. Acta Endocrinol (Copenh) **121:**651–658.
31. Ueda S, Takamatsu J, Fukata S, Tanaka K, Shimizu N, Sakata S, Yamaji T, Kuma K, Ohsawa N 1996 Differences in response of thyrotropin to 3,5,3′-triiodothyronine and 3,5,3′-triiodothyroacetic acid in patients with resistance to thyroid hormone. Thyroid **6:**563–570.

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CLINICAL REPORT

AMERICAN JOURNAL OF medical genetics PART A

First Case of a Japanese Girl With Myhre Syndrome Due to a Heterozygous *SMAD4* Mutation

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This article reports the first case of a Japanese girl with molecularly confirmed Myhre syndrome (MS). The patient was 9 years old at her first visit, and she had been diagnosed with unknown skeletal dysplasia. Her phenotype fulfilled the clinical and radiological criteria for MS, such as typical facies with prognathism, hearing impairment, short stature, square body shape, and limited joint mobility. The thick calvarium and thick skin were clues to the clinical diagnosis of MS. A heterozygous mutation in the mothers-against-DPP homolog 4 (*SMAD4*) gene has been reported to cause MS. We sequenced *SMAD4* using standard PCR-based technique and identified a recurrent mutation (p.Ile500 Thr). She attained menarche before 11 years of age; however, she developed oligomenorrhea after a few years of 40-day cycles, necessitating hormone replacement therapy. The luteinizing hormone-releasing hormone (LHRH) tests suggested abnormalities related to hypothalamo-hypophyseal malfunction. Previous reports on MS described early menarche in girls and early or delayed puberty and cryptorchidism in boys. Therefore, we recommend performing an endocrinological evaluation of the hypothalamo-hypophyseal-gonadal axis in patients with MS to clarify whether hormonal abnormalities are associated with the syndrome.

Key words: Myhre syndrome; *SMAD4*; growth retardation; thick calvarium; muscular hypertrophy; gonadal dysfunction

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INTRODUCTION

Myhre syndrome (MS, OMIM 139210) is a rare connective tissue disorder and was first described by Myhre et al. [1981] in two unrelated males in 1981. Since then, fewer than 30 individuals with MS have been reported till date [Soljak et al., 1983; Garcia-Cruz et al., 1993; Titomanlio et al., 2001; Whiteford et al., 2001; Burglen et al., 2003; Dávalos et al., 2003; Lopez-Cardona et al., 2004; Rulli et al., 2005; Van Steensel et al., 2005; Becerra-Solano et al., 2008]. The clinical hallmarks of MS include intellectual disability; low birth weight; poor postnatal growth leading to short stature; conductive and sensorial hearing loss; muscular hypertrophy; limited joint mobility; and typical facies characterized by distinct prognathism, blepharophimosis, and a narrow mouth. X-ray findings of MS include a thick calvarium, mandibular protrusion, mild rib broadening, hypoplastic iliac wings, shortening of the tubular bones, and somewhat flattened vertebral bodies with large, short pedicles. Two recent studies have carried out exome sequencing of individuals with MS and reported that heterozygous missense mothers-against-decapentaplegic homolog of 4 (*SMAD4*; NM 005359) mutations affect the codon for Ile500 in all the study subjects (n = 19) [Caputo et al., 2012; Le Goff et al., 2012]. We sequenced the *SMAD4* gene using standard PCR-based technique and identified a recurrent mutation (p.Ile500 Thr) in our patient. Early menarche in girls and early or delayed puberty and cryptorchidism in boys have been reported in MS. Our patient also complained of oligomenorrhea. In this article, we report on a case of molecularly confirmed MS and hormonal evaluations of the patient.

CLINICAL REPORT

We obtained written informed consent from the patient and her parents for molecular studies and publication of her clinical

Additional supporting information may be found in the online version of this article.

Conflicts of interest: None.

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photographs. This study was approved by the institutional review board of Kanagawa Children's Medical Center, Kanagawa, Japan.

The patient is a Japanese girl who is currently 18 years old. She was the first child born to unrelated healthy parents after a full-term pregnancy. At birth, she weighed 2,416 g and was 47 cm tall. Soon after birth, choanal atresia, membranous cleft palate, and limited joint mobility were noted. Early developmental milestones, especially speech, were delayed, and audiological evaluation revealed bilateral hearing loss. Her younger sister is healthy.

At the age of 9 years, she presented with painful hip joint of the right. She was diagnosed with avascular necrosis of the right femoral head. Multiple joint contractures and short stature were noted at that time. She was referred to us for investigating her short stature at the age of 10 years. She weighed 35.0 kg and was 118.5 cm (−2.8 SD) tall, and her BMI was 24.9. She had rhizomelic shortening of the limbs, distinct square body shape with hypertrophic extremities (Fig. 1A), and brachydactyly (Fig. 1B). Her skin was thick and stiff. She had a flat wide facies with prominent prognathism, blepharophimosis, and a narrow mouth with a thin vermilion of the upper lip (Fig. 1A). Audiological examination revealed mixed conductive and sensorial hearing loss (right: 67 dB, left: 87 dB). The patient had hyperopia and astigmatism, and right side amblyopia. Radiological examination revealed a thick calvarium, mandibular protrusion (Fig. 1D), shortening of the tubular bones, and large pedicles and thick neural arches, resulting in a narrow spinal canal. MRI showed thick basilar bone and large and thick clivus. Spinal fluid space at the C1 level was very narrow (Fig. 1E).

She attained menarche at 10 years and 10 months and gained 40 day-cycles 2 years later. At the age of 15, she developed oligomenorrhea, necessitating hormone replacement therapy. Her adult height is 127.0 cm (−5.8 SD).

The patient was diagnosed with skeletal dysplasia of unknown origin and treated for this condition for several years, after which MS became a potential candidate. The thick calvarium and thick and stiff skin were clues to the diagnosis of MS. Table I presents a comparison of clinical features of this patient with those of previously reported cases (clinically diagnosed cases and molecularly confirmed cases with *SMAD4* mutations). She fulfilled the clinical criteria reported for MS.

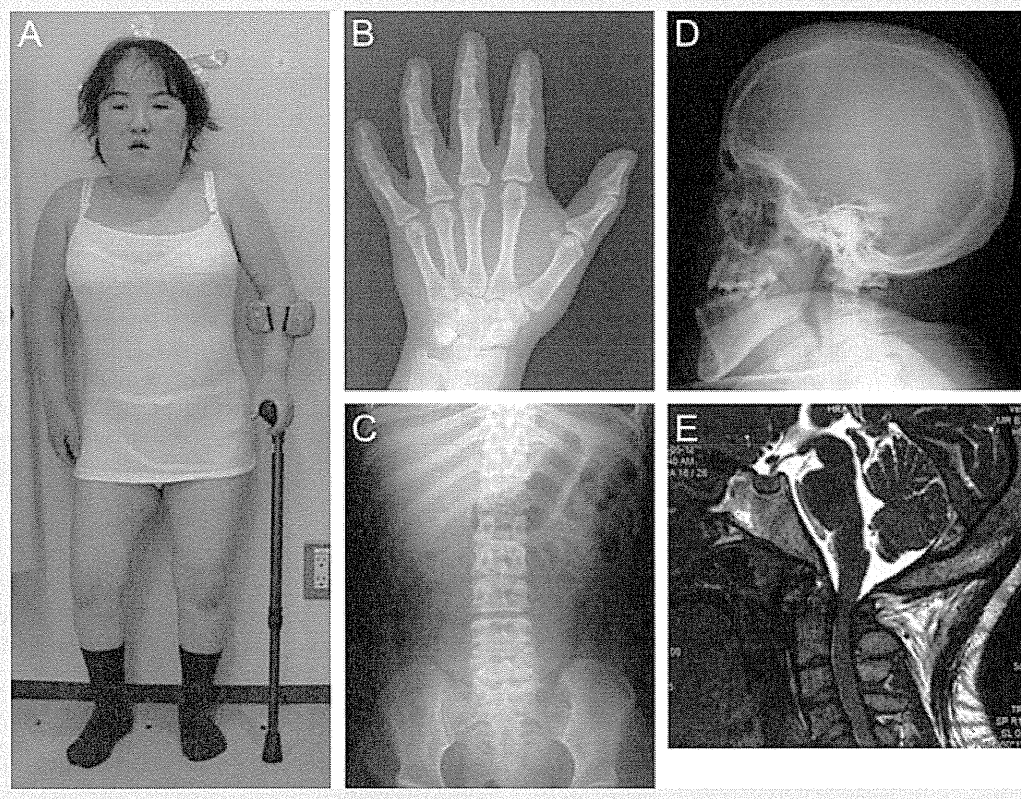

FIG. 1. Clinical manifestations of Myhre syndrome in our patient (A). Note the rhizomelic shortening of the limbs, impressive square body shape, flat wide facies with distinct prognathism, deep-set eyes, short palpebral fissures, and narrow mouth with thin upper lip (A). Skeletal manifestations of the patient are shown (B–E). Note brachydactyly (B), the broad ribs, hypoplastic iliac wings, and platyspondyly with large pedicles (C), thick calvarium, prognathism (D), thick basilar bone and large and thick clivus, and unidentified spinal fluid space at the C1 level detected on T2-weighted MRI scanning (0.8-mm slice) (E).

TABLE I. Comparison of the Clinical and Radiological Features of the Current Case With Those of Previously Reported Patients With Clinically Diagnosed Myhre Syndrome and Recently Confirmed Cases With *SMAD4* Mutations

	Clinically diagnosed Myhre syndrome	Myhre syndrome with *SMAD4* mutation	Current case
Clinical features			
Short stature	16/16	18/19	+
Intellectual disability	14/16	17/19	+
Muscular build	14/16	19/19	+
Decreased joint mobility	14/15	19/19	+
Thick skin	10/12	15/19	+
Brachydactyly	16/16	19/19	+
Deafness	13/16	16/17	+
Midfacial hypoplasia	14/15	8/8	+
Blepharophimosis	15/16	18/19	+
Short philtrum	14/16	18/19	+
Narrow mouth and/or thin vermilion of the upper lip	14/16	6/8	+
Prognathism	14/14	19/19	+
Cardiac anomalies	7/16	14/19	−
Cryptorchidism	5/10	2/5	
Precocious puberty	4/8		?
Premature menarche	2/5	5/8	−
Secondary amenorrhea		1/3	+
Delayed puberty	3/8		−
Radiological features			
Thick calvarium	14/16	17/17	+
Broad ribs	12/14	4/8	+
Narrow pelvis	15/15	8/8	+
Mild platyspondyly	12/14	10/17	+
Large pedicles	10/14	15/16	+

Laboratory and Endocrinological Assessment

Results of the routine biochemical studies were within the normal ranges. Results of the screening tests for metabolic defects, including analysis of urinary mucopolysaccharides and blood amino acids were normal. Routine karyotyping (G-bands) showed 46,XX, and the results of the array CGH (Agilent SurePrint G3 60K) were also negative. Skin histology showed moderately collagenized dermis with unremarkable epidermis. EMG showed no abnormalities.

Endocrinological studies (basal thyroid profile, LH, FSH, estradiol, cortisol, and IGF-1 serum determinations) showed normal results. The patient underwent a provocative test by insulin-induced hypoglycemia (ITT), thyrotropin-releasing hormone (TRH), and luteinizing hormone-releasing hormone (LHRH), and the results are shown in Table II. Although the peak GH response was $<10\ \mu g/L$, which is the cutoff for GH deficiency (GHD), the serum IGF-1 level was appropriate for a pubertal girl. This finding indicated that her GH secretion was not defective. Gonadotropin responses were not excessive or inappropriate for an early pubertal girl. To evaluate gonadal function, LHRH test was repeated when the patient was 18 years of age. The basal LH and follicle-stimulating hormone (FSH) levels were unchanged, and the peak LH (23.9 IU/L) and peak FSH (15.49 IU/L) levels were slightly higher and lower than the corresponding levels in this patient at 10 years of age, respectively. The free testosterone level (2.1 pmol/L) in the serum was not elevated. A pelvic ultrasound (US) study revealed that the uterus and left ovary were normal for the patient's age, but the right ovary was slightly small. No signs of polycystic ovary syndrome were identified.

Molecular Analyses

We extracted genomic DNA from peripheral lymphocytes using a standard technique. Molecular screening for geleophysic dysplasia (GD, OMIM 231050) and acromicric dysplasia (AD, MIM 102370), direct sequence analysis of the 18 coding exons of adamts-like protein 2 (*ADAMTSL2*) [Le Goff et al., 2008] and transforming growth factor β (TGFβ) binding-protein like domain 5 of fibrillin 1 (*FBN1*) (exons 41 and 42) [Le Goff et al., 2011] revealed no significant mutations causing amino acid alterations. All the 11 coding exons of *SMAD4* were PCR amplified and sequenced as reported previously [Le Goff et al., 2011]. A heterozygous *SMAD4* mutation (c1499T>C, p.Ile500 Thr) was identified in our patient (Supplementary eFig. 1—see Supporting Information online), which was one of the mutations described previously [Le Goff et al., 2011; Caputo et al., 2012].

DISCUSSION

This report is the first to describe the clinical course of a Japanese patient with a heterozygous *SMAD4* mutation. The patient exhibited a full MS phenotype of the clinical and radiological criteria

TABLE II. Hormonal Evaluation of the Patient at 10 Years of Age

Min	0	15	30	60	90	120
Provocation test by insulin-induced hypoglycemia, luteinizing hormone-releasing hormone (LH-RH), and thyrotropin-releasing hormone (TRH)						
BS (mmol/L)	4.16	2.83	3.22	3.66	4.00	4.16
GH (μg/L)	6.21	1.8	1.07	7.24	5.47	3.33
LH (IU/L)	1.4	16.2	18.3	15.5	12.0	12.0
FSH (IU/L)	8.8	18.2	21.8	23.0	23.0	23.9
TSH (mU/L)	3.0	16.1	17.0	13.3	8.6	5.7
ACTH (pmol/L)	5.59	5.64	7.29	9.60	9.01	8.04
Cortisol (nmol/L)	240	171	146	488	331	246

GH, growth hormone; LH, luteinizing hormone; FSH, follicle stimulating hormone; TSH, thyroid-stimulating hormone; ACTH, adrenocorticotropic hormone; BS, blood glucose.
Basal hormonal data: IGF-1 = 240 μg/L; IGFBP-3 = 2.23 μg/L; E2 = 91.0 pmol/L; FT3 = 6.19 pmol/L; FT4 = 17.9 pmol/L.
IGF-1, insulin-like growth factor-1; IGFBP-3, insulin-like growth factor-binding protein-3; FT3, free thyronine; FT4, free thyroxine; E2, estradiol.

reported in MS, including typical facies with distinct prognathism, short stature, limited joint mobility and square body shape, stiff skin, and typical radiological findings (thick calvarium, distinctive mandibular protrusion, hypoplastic iliac wings, shortening of the tubular bones, and large pedicles and thick neural arches) [Burglen et al., 2003; Becerra-Solano et al., 2008]. Spinal canal stenosis at the craniovertebral junction was the finding that did not previously attract attention.

On clinical grounds, the phenotype of MS overlaps with that of a few syndromes. growth retardation, ocular abnormalities, microcephaly, brachydactyly, and oligophrenia syndrome (GOMBO) was previously confused with MS, but is currently known to be caused by a cryptic translocation between chromosome 3p and 22q [Verloes et al., 2000]. Since the array CGH (Agilent SurePrint G3 60K) analysis revealed no abnormal findings, this translocation could be excluded. Another disorder that attracts attention is laryngotracheal stenosis, arthropathy, prognathism, and short stature syndrome (LAPS). However, none of the individuals with MS have had recurrent laryngotracheal stenosis, which is a hallmark of LAPS. Apart from this, the other findings are sufficiently similar between the two syndromes, and Lindor [2009] speculated that the two syndromes might represent the same entity, but the nosological esteem of LAPS remains elusive.

Additionally AD, GD, and Weill–Marchesani syndrome (WMS, OMIM 608328) deserve detailed comments. Since AD, GD, and WMS share common clinical findings, such as short stature, joint limitations, brachydactyly, and skin thickness, and similar radiological findings, the probable pathogenic link among these disorders has been discussed. Recently, *ADAMTSL2* mutations have been identified in GD patients [Allali et al., 2011], and *FBN1* mutations located in exons 41 and 42 have been identified in *ADAMTSL2*-negative GD and AD patients [Le Goff et al., 2011]. *FBN1* mutations are also responsible for WMS [Faivre et al., 2003]. The results of the molecular screening of *ADAMTSL2* and *FBN1* supported the differential diagnosis. The most recent investigations based on the whole exome strategy revealed that MS is attributed to domain-specific, heterozygous missense mutations in *SMAD4*. Direct sequence analysis of the coding lesions led to the identification of three missense mutations in the region of *SMAD4* coding for the MH2 domain, all affecting an isoleucine residue at position 500 (p.Ile500 Thr, p.Ile500 Val, and p.Ile500 Met) in all the 19 study subjects [Caputo et al., 2012; Le Goff et al., 2012]. *SMAD4* on chromosome 18q21.2 has been established as a tumor suppressor gene. Inactivation of *SMAD4* has been demonstrated in cases of pancreatic and colorectal carcinoma [Hahn et al., 1996; Schutte et al., 1996]. Loss-of-function lesions and deletions have been documented in juvenile polyposis syndrome [Howe et al., 1998]. *SMAD4* is known as a transducer mediating TGFβ and bone morphogenic pathway (BMP) signaling. The previous findings of enhanced TGFβ signaling in GD and AD [Le Goff et al., 2011] and the recent findings of decreased expression of downstream TGFβ target genes [Le Goff et al., 2012] support the idea that MS, AD, GD, and WMS constitute a group of disorders related to impaired TGFβ signaling. The mechanisms for each of the characteristic but homogenous phenotypic features found in MS have not yet been clarified; however, several findings support the role of *Smad4* in MS. The abrogation of *Smad4* in chondrocytes resulted in dwarfism with severely disorganized growth palate in mice [Zhang et al., 2005]. Mice with conditional *Smad4* knockout in chondrocytes are characterized by smaller cochlear volume, bone malformation, and abnormalities of osseous spiral lamina and basilar membrane have been reported to lead to severe sensorineural hearing loss in mice [Yang et al., 2009]. These observations suggest that loss of function of *SMAD4* may be essential in the pathogenesis of MS.

Girls with early menarche and boys with early or delayed puberty and cryptorchidism have been previously reported in patients with MS (Table I). Of the eight female patients with *SMAD4* mutation, five have had premature menarche [Le Goff et al., 2011] and of the five male patients with *SMAD4* mutation, two had cryptorchidism [Caputo et al., 2012]. From these findings, we believe that endocrinological abnormalities related to the hypothalamo-hypophyseal-gonadal axis should be evaluated and discussed as part of the syndrome. Although the exact age of puberty is not known, our patient showed secondary sexual development at her first visit and had her first period just before she was 11 years of age. These observations suggested that her secondary sexual development would have been within the normal range. Thereafter, she became oligomenorrhic and hormone replacement therapy was necessary when she was 15 years. Polycystic ovary syndrome seemed

unlikely because the ratio of serum LH/FSH was less than 1, there were low levels of free testosterone in the serum, and the ovarian morphology by echogram appeared normal. The LHRH test was repeated when she was 18 years old, and the test results suggested menstrual malfunction without excess gonadotropin, and the abnormalities were not related to the ovaries but possibly to the disturbance of the hypothalamo-hypophyseal-gonadal axis. Secondary amenorrhea has been reported in a previous case with *SMAD4* mutation, but no observations related to the menstrual cycles were recorded in previous reports. Although it remains unknown how *SMAD4* mutations identified in MS affect gonadal function, we propose that endocrinological evaluation of the hypothalamo-hypophyseal-gonadal axis should be considered in patients with MS to clarify whether it is associated with the syndrome.

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REFERENCES

Allali S, Le Goff C, Pressac-Diebold I, Pfennig G, Mahaut C, Dagoneau N, Alanay Y, Brady AF, Crow YJ, Devriendt K, Drouin-Garraud V, Flori E, Geneviève D, Hennekam RC, Hurst J, Krakow D, Le Merrer M, Lichtenbelt KD, Lynch SA, Lyonnet S, MacDermot K, Mansour S, Megarbané A, Santos HG, Splitt M, Superti-Furga A, Unger S, Williams D, Munnich A, Cormier-Daire V. 2011. Molecular screening of *ADAMTSL2* gene in 33 patients reveals the genetic heterogeneity of geleophysic dysplasia. J Med Genet 48:417–421.

Becerra-Solano LE, Díaz-Rodriguez M, Nastasi-Catanese JA, Toscano-Flores JJ, Banuelos-Robles O, Figuera LE, Matute E, de Lourdes Ramírez-Duenas M. 2008. The fifth female patient with Myhre syndrome: Further delineation. Clin Dysmorphol 17:113–117.

Burglen L, Héron D, Moerman A, Dieux-Coeslier A, Bourguignon JP, Bachy A, Carel JC, Cormier-Daire V, Manouvrier S, Verloes A. 2003. Myhre syndrome: New reports, review, and differential diagnosis. J Med Genet 40:546–551.

Caputo V, Cianetti L, Niceta M, Carta C, Ciolfi A, Bocchinfuso G, Carrani E, Dentici ML, Biamino E, Belligni E, Garavelli L, Boccone L, Melis D, Andria G, Gelb BD, Stella L, Silengo M, Dallapiccola B, Tartaglia M. 2012. A restricted spectrum of mutations in the *SMAD4* Tumor-Suppressor gene underlies Myhre syndrome. Am J Hum Genet 90:161–169.

Dávalos NO, Garcia-Ortiz JE, Garcia-Cruz D, Feria-Velasco A, Sánchez-Corona J. 2003. Myhre syndrome: First female case. Clin Dysmorphol 12:119–121.

Faivre L, Gorlin RJ, Wirtz MK, Godfrey M, Dagoneau N, Samples JR, Le Merrer M, Collod-Beroud G, Boileau C, Munnich A, Cormier-Daire V. 2003. In frame fibrillin-1 gene deletion in autosomal dominant Weill-Marchesani syndrome. Am J Med Genet 40:204–207.

Garcia-Cruz D, Figuera LE, Feria-Velazco A, Sánchez-Corona J, Garcia-Cruz MO, Ramirez-Duenás RM, Hernandez-Córdova A, Ruiz MX, Bitar-Alatorre WE, Ramirez-Duenas ML, Cantú JM. 1993. The Myhre syndrome: Report of two cases. Clin Genet 44:203–207.

Hahn SA, Schutte M, Hoque AT, Moskaluk CA, da Costa LT, Rozenblum E, Weinstein CL, Fischer A, Yeo CJ, Hruban RH, Kern SE. 1996. DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. Science 271:350–353.

Howe JR, Roth S, Ringold JC, Summers RW, Jarvinen HJ, Sistonen P, Tomlinson IP, Houlston RS, Bevan S, Mitros FA, Stone EM, Aaltonen LA. 1998. Mutations in the SMAD4/DPC4 gene in juvenile polyposis. Science 280:1086–1088.

Le Goff C, Morice-Picard F, Dagoneau N, Wang LW, Perrot C, Crow YJ, Bauer F, Flori E, Prost-Squarcioni C, Krakow D, Ge G, Greenspan DS, Bonnet D, Le Merrer M, Munnich A, Apte SS, Cormier-Daire V. 2008. *ADAMTSL2* mutations in geleophysic dysplasia demonstrate a role for ADAMTS-like proteins in TGF-beta bioavailability regulation. Nat Genet 40:1119–1123.

Le Goff C, Mahaut C, Wang LW, Allali S, Abhyankar A, Jensen S, Zylberberg L, Collod-Beroud G, Bonnet D, Alanay Y, Brady AF, Cordier MP, Devriendt K, Genevieve D, Kiper PO?, Kitoh H, Krakow D, Lynch SA, Le Merrer M, Mégarbane A, Mortier G, Odent S, Polak M, Rohrbach M, Sillence D, Stolte-Dijkstra I, Superti-Furga A, Rimoin DL, Topouchian V, Unger S, Zabel B, Bole-Feysot C, Nitschke P, Handford P, Casanova JL, Boileau C, Apte SS, Munnich A, Cormier-Daire V. 2011. Mutations in the TGFβ binding-protein-like domain 5 of *FBN1* are responsible for acromicric and geleophysic dysplasias. Am J Hum Genet 89:7–14.

Le Goff C, Mahaut C, Abhyankar A, Le Goff W, Serre V, Afenjar A, Destrée A, di Rocco M, Héron D, Jacquemont S, Marlin S, Simon M, Tolmie J, Verloes A, Casanova JL, Munnich A, Cormier-Daire V. 2012. Mutations at a single codon in Mad homology 2 domain of SMAD4 cause Myhre syndrome. Nat Genet 44:85–88.

Lindor NM. 2009. LAPS syndrome and Myhre syndrome: Two disorders or one? Am J Med Genet Part A 149A:798–799.

Lopez-Cardona MG, Garcia-Cruz D, Garcia-Ortiz JE, Davalos NO, Feria-Velasco A, Rodriguez-Rojas LX, Garcia-Cruz MO, Figuera-Villanueva LE, Stephens A, Larios-Arceo F, Sanchez-Corona J. 2004. Second female case of Myhre syndrome. Clinl Dysmorphol 13:91–94.

Myhre SA, Ruvalcaba RH, Graham CB. 1981. A new growth deficiency syndrome. Clin Genet 20:1–5.

Rulli I, Ferrero GB, Belligni E, Delmonaco AG, Defilippi C, Silengo M. 2005. Myhre's syndrome in a girl with normal intelligence. Am J Med Genet Part A 134A:100–102.

Schutte M, Hruban RH, Hedrick L, Cho KR, Nadasdy GM, Weinstein CL, Bova GS, Isaacs WB, Cairns P, Nawroz H, Sidransky D, Casero RA Jr, Meltzer PS, Hahn SA, Kern SE. 1996. DPC4 gene in various tumor types. Cancer Res 56:2527–2530.

Soljak MA, Aftimos S, Gluckman PD. 1983. A new syndrome of short stature, joint limitation and muscle hypertrophy. Clin Genet 23:441–446.

Titomanlio L, Marzano MG, Rossi E, D'Armiento M, De Brasi D, Vega GR, Andreucci MV, Orsini AV, Santoro L, Sebastio G. 2001. Case of Myhre syndrome with autism and peculiar skin histological findings. Am J Med Genet 103:163–165.

Van Steensel MA, Vreeburg M, Steijlen PM, de Die-Smulders C. 2005. Myhre syndrome in a female with previously undescribed symptoms: Further delineation of the phenotype. Am J Med Genet Part A 139A:127–130.

Verloes A, Lesenfants S, Jamar M, Dideberg V, Herens C. 2000. GOMBO syndrome: Another "pseudorecessive" disorder due to a cryptic translocation. Am J Med Genet 95:185–186.

Whiteford ML, Doig WB, Raine PA, Hollman AS, Tolmie JL. 2001. A new case of Myhre syndrome. Clin Dysmorphol 10:135–140.

Yang SM, Hou ZH, Yang G, Zhang JS, Hu YY, Sun JH, Guo WW, He D, Han DY, Young WY, Yang X. 2009. Chondrocyte-specific Smad4 gene conditional knockout results in hearing loss and inner ear malformation in mice. Dev Dyn 238:1897–1908.

Zhang J, Tan X, Li W, Wang Y, Wang J, Cheng X, Yang X. 2005. Smad4 is required for the normal organization of the cartilage growth plate. Dev Biol 15:311–322.

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CLINICAL STUDY

Mass screening of newborns for congenital hypothyroidism of central origin by free thyroxine measurement of blood samples on filter paper

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Abstract

Objective: To evaluate the effectiveness of mass screening of newborns for congenital hypothyroidism of central origin (CH-C) by measurement of free thyroxine (FT_4) and thyroid-stimulating hormone (TSH).
Design: Questionnaire-based survey of CH-C patients born between 1999 and 2008 in Kanagawa prefecture, Japan.
Methods: TSH and FT_4 levels in dried blood spots on filter paper were measured using ELISA kits, and CH-C was diagnosed at FT_4 levels below a cutoff of 0.7 ng/dl (9.0 pmol/l). Survey results were collated with the database created by the screening organizer.
Results: Twenty-four CH-C patients (18 males) were identified, 14 of whom had multiple pituitary hormone deficiencies (group M), eight had isolated CH-C (group I), and two had undetermined pituitary involvement (group U). In groups M, I, and U, the number of patients with FT_4 levels below the cutoff value at screening was five (36%), seven (88%), and one (50%) respectively; other patients had been diagnosed clinically. Thus, 13 patients were true positives, while nine were false negatives, yielding screening sensitivity of 59.1% and positive predictive value of 11.5%. The calculated sensitivity was 81.8% at a higher cutoff value of 0.9 ng/dl (11.6 pmol/l). The overall incidence of CH-C was estimated at 1 in 30 833 live births, while that of CH of thyroidal origin (CH-T) is 1 in 3472 live births in Kanagawa prefecture (CH-T/CH-C, 8.9).
Conclusions: Newborn screening with combined FT_4 and TSH measurements can identify a significant number of CH-C patients before manifestation of clinical symptoms, but a more appropriate FT_4 cutoff value should be considered.

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Introduction

Screening of newborns for congenital hypothyroidism (CH) is now routinely used in most of the developed world and in an increasing number of developing countries, which has prevented serious intellectual sequelae in a considerable number of patients with CH (1, 2). While most CH cases are due to CH of thyroidal origin (CH-T) manifesting as thyroid dysgenesis or thyroid hormone synthesis defects, a significant number of CH cases are due to inadequate thyroid-stimulating hormone (TSH) secretion from the anterior pituitary (3, 4, 5, 6, 7, 8, 9). The latter category of CH cases is termed as CH of central origin (CH-C). The incidence of CH-C is estimated to be ~1 in 20 000–30 000 live births (3, 5, 6, 7, 10), which is much higher than previously thought. Nevertheless, CH screening in Japan is mainly based on the detection of elevated TSH levels in dried blood samples on filter paper (primary TSH strategy). This assay has demonstrated high sensitivity in detecting CH-T (11, 12) but failed to identify newborns with CH-C. On the other hand, screening based on the detection of low T_4 levels (primary T_4 strategy) can identify CH-C newborns only inefficiently, as false-positive cases are inevitable due to both thyroxine-binding globulin (TBG) deficiency and transient low T_4 levels in critically ill newborns.

To overcome this situation, The Netherlands has implemented a system of assaying TSH, T_4, and TBG, which can eliminate false-positive results caused by TBG deficiency (5, 6). Assaying free T_4 (FT_4) may be an alternative solution because FT_4 is less influenced by TBG than T_4. Moreover, determination of FT_4 seems to be superior to that of T_4 because this reduces false-positive cases in premature newborns, according to the report of a smaller difference between full-term and

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preterm newborns in FT_4 levels than in T_4 levels measured in dried blood samples on filter paper (13). Therefore, in Kanagawa prefecture, we have adopted a strategy of simultaneously measuring TSH and FT_4 in all newborns using a filter paper assay (9). Sapporo city has also adopted the same screening system. The report of a 5-year audit in Sapporo city was released in 2004, in which six CH-C cases were identified through this screening (7). However, the study in Sapporo included only patients showing positive screening results, which preclude evaluation of the sensitivity of screening in detecting CH-C. In addition, the annual birth rate in Sapporo is approximately one-fourth of Kanagawa prefecture.

To evaluate the effectiveness of our CH-C screening system, we have conducted a detailed, comprehensive survey of CH-C patients from Kanagawa region, Japan. In this study, all CH-C cases detected via screening and diagnosed clinically were included and used to estimate the sensitivity and positive predictive value (PPV) of the screening method.

Subjects and methods

Outline of newborn screening system

Kanagawa prefecture, in which Yokohama is the main city, is located in the central region of the Japanese islands, neighboring the Tokyo metropolitan area. The annual number of births in Kanagawa prefecture has been ~70 000 in recent years. The incidence of CH-T in Kanagawa prefecture is estimated to be 1 in 3472 births. Neonatal screening is exclusively conducted by the Neonatal Mass-screening Committee (NMC) of the Kanagawa Prefecture Medical Association (KPMA), which comprises executive officers, technical experts, gynecologists, general pediatricians, and pediatric endocrinologists. The screening procedure adopted by the NMC-KPMA is based on the determination of TSH and FT_4 in dried blood spots on filter paper obtained 4 to 7 days after birth (median sampling day was the fifth day). According to the standard practice followed, newborns with high TSH levels ($\geq$30 μIU/ml serum) are immediately sent to one of the several pediatric endocrine units within the prefecture. A second filter paper sampling is requested for those with borderline TSH levels (15–30 μIU/ml serum) or low FT_4 levels ($<$0.7 ng/dl of serum (9.0 pmol/l)). If the results again indicate borderline TSH or low FT_4, the baby is sent for a thorough evaluation. Thus, CH-C is suspected if FT_4 levels are low in two consecutive samples. To eliminate cases with transient low FT_4 due to prematurity, samples taken from the newborns with birth weight $<$2000 g are considered to be preliminary, and the results are sent to each attending physician as an unofficial report. Once the baby attains a weight of 2500 g or reaches 30 days of age, the first sample is requested.

TSH levels in filter paper samples were determined by ELISA using mouse monoclonal antihuman TSH antibodies (Eiken Chemical Co. Ltd., Tochigi, Japan). To determine FT_4 levels in filter paper samples, ENZAPLATE N-FT_4 was used (Siemens Healthcare Diagnostics K.K., Tokyo, Japan), which is an ELISA kit based on a competitive reaction between sample FT_4 and peroxidase-tagged human T_4 to bind to rabbit polyclonal antihuman T_4 antibody (first antibody). A 3 mm disc is punched out from the filter paper and is incubated with peroxidase-tagged T_4 and the first antibody in a reaction mixture of 150 μl for 4 h at 18–25 °C in a micro-well plate with immobilized caprine antirabbit IgG antibodies (second antibody). After removal of the filter paper disc and washing five times, *O*-phenylenediamine is added, and the absorbance is then measured at 492 nm. A calibration curve is established using standard filter paper samples of known FT_4 concentrations, which are provided by the manufacturer. FT_4 level in the sample is then determined by comparison with the calibration curve.

The performance of this kit, of which there is only one study, reported in a Japanese journal (14), is as follows. The FT_4 determination range is 0.5–5.0 ng/dl, which is based on a precision level lower than 15% of the coefficient of variation (CV). Intra-assay CV is 7.6–15.0%, whereas inter-assay CV is 9.4–18.5%. The correlation between the FT_4 levels measured by this kit and the electrochemiluminescence immunoassay (ECLIA) kit (Elecsys FT_4; Roche Diagnostics) is shown in Fig. 1.

Preliminary survey

A preliminary survey was conducted in December 2008. Questionnaires were sent to all 139 hospitals with a pediatric section in Kanagawa prefecture. The questionnaire included questions about the number of CH-C patients born in Kanagawa prefecture between January 1999 and December 2008 and treated continuously with levothyroxine (L-T_4). CH-C was defined as CH considered to be of hypothalamic or pituitary origin, excluding acquired sequelae of head trauma, brain tumor, etc., and irrespective of involvement of other pituitary functions. Cases of hypothyroxinemia due to prematurity were excluded.

Secondary survey

In April 2009, we requested the corresponding doctors caring for the probable CH-C patients identified in the preliminary survey to provide detailed information, including patient profile, medical complications, data on newborn screening, and results of thyroid function, thyroid imaging studies, and pituitary function tests with imaging information.

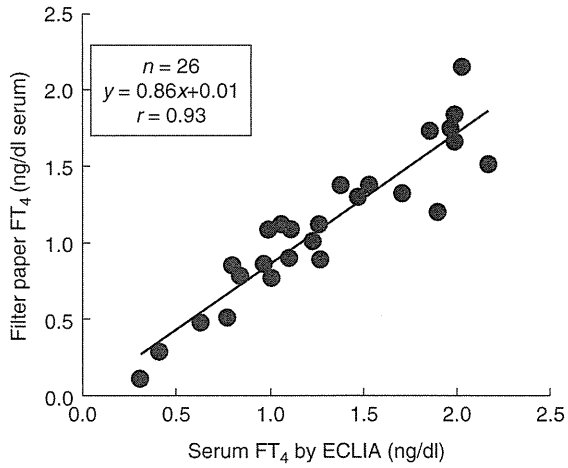

Figure 1 Correlation between FT_4 levels in dried blood samples on filter paper measured by ELISA and FT_4 serum levels measured by ECLIA for newborns and young infants. Filter paper blood specimens and serum samples were collected simultaneously from 26 infants younger than 2 months. FT_4 levels of blood samples on the filter paper were measured by an ELISA kit, whereas serum FT_4 was measured by ECLIA.

Collation study

After completion of the secondary survey, we collated the list of CH-C patients identified through the above surveys with the NMC-KPMA database, in which information from the first-line investigation at the pediatric endocrine unit and the screening results for all patients with positive screening results had been compiled.

Patient categorization

CH-C patients identified through the secondary survey and collation study were categorized into three groups according to the involvement of other pituitary hormones. Group M comprised CH-C patients with at least one pituitary hormone deficiency other than insufficient TSH secretion. These patients were considered to have congenital hypopituitarism with multiple pituitary hormone deficiencies. The diagnosis of each pituitary hormone deficiency was based on the attending physician's evaluation, except for GH deficiency, which was verified by at least one pharmacological stimulation test. Group I comprised isolated CH-C patients without pituitary involvement other than TSH insufficiency. Group U consisted of CH-C patients for whom pituitary involvement was undetermined.

Statistical analysis

Statistical analysis was carried out using Microsoft Office Excel 2007 (Microsoft Corporation). Correlation between the assay results of FT_4 (ELISA) in filter paper samples and serum FT_4 (ECLIA) was evaluated by linear regression analysis. Mann–Whitney *U*-test was used to compare FT_4 values between groups M and I. Fisher's exact probability test was used to compare the incidence of screening positive patients according to the etiological categories (groups M and I). *P* values of <0.05 were considered to be significant.

The Ethics Committee of Kanagawa Children's Medical Center reviewed and approved the study procedures.

Results

Out of the 139 hospitals from Kanagawa prefecture to which the preliminary survey questionnaire was sent, responses were obtained from 94 hospitals, including 14 hospitals stating that they currently had no pediatric section. Accordingly, the actual response rate was calculated to be 64.0% (80/125 hospitals with pediatric sections). Through this primary survey, 42 patients with probable CH-C (2–11 years old) were identified at 14 out of the 80 hospitals.

Figure 2 shows the number of CH-C patients, both probable and confirmed, identified through the surveys. The preliminary survey identified 42 probable patients, of which 20 patients were considered to represent true CH-C cases. After collation with the NMC-KPMA database, 24 CH-C patients (of which 18 were male) were finally identified. Details of each patient are summarized in Tables 1 and 2. As the total number of newborns screened during the study period was 740 003, we calculated the minimal incidence of

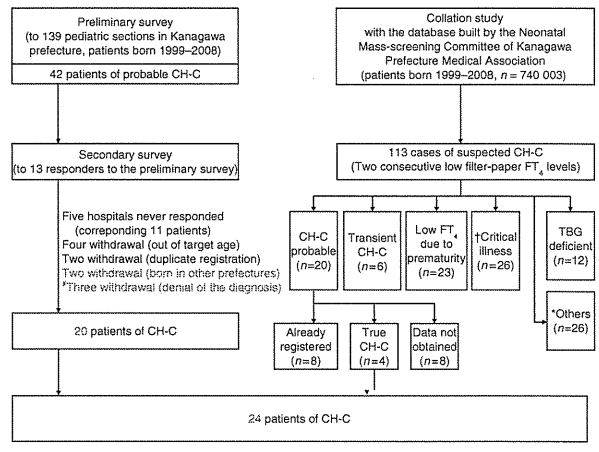

Figure 2 Overview of the study. #Three patients were excluded because they were judged not to have CH-C. Of these, two patients were diagnosed with CH-T with delayed TSH elevation, while the third patient had transient low FT_4, possibly because of an emotional deprivation syndrome. †Including one newborn with hydranencephaly. *Patients from whom we could not obtain detailed information.

Table 1 Characteristics of 14 patients with CH-C with multiple pituitary hormone deficiencies (group M). Patient 13 was already on L-thyroxine treatment at the time of screening.

	At birth			FT_4 values at Sc (ng/dl)		At first presentation			
Pt. no., sex	Year	Wt (g)	**Diagnostic symptom** (age)	1st sample (day)	2nd sample (day)	Serum TSH (μIU/ml)	Serum FT_4 (ng/dl)	**Deficient pituitary hormones**	**MR imaging of the CN**
1, F	1999	2872	Low vision (4M)	0.90 (5)		1.59	0.86	TSH, GH, AVP	APS, EPP, ONH, ASP
2, F	2000	3352	SS (1Y)	1.42 (4)		4.34	0.75	TSH, GH, LH/FSH	APS, EPP, PH
3, F	2000	2994	SS (4Y)	1.80 (5)		0.79	0.84	TSH, GH, AVP	Normal
4, M	2000	4420	Icterus (2M)	0.81 (26)		2.90	1.00	TSH, GH, ACTH	APS, EPP, ONH, ASP
5, M	2001	3240	Shock (1D)	0.83 (7)		7.40	0.70	TSH, GH, ACTH, LH/FSH	APS, EPP, ONH
6, F	2002	3150	Shock (1D)	0.58 (5)		10.28	0.65	TSH, GH, ACTH, LH/FSH	APS, EPP, ONH
7, M	2002	2342	Seizure (1Y)	0.81 (7)		1.71	0.42	TSH, GH, ACTH	APS, EPP, PH
8, M	2004	2275	Sc (23D)	0.48 (5)	0.50 (23)	3.77	0.76	TSH, ACTH	Normal
9, M	2005	3135	Sc (22D)	0.55 (5)	0.38 (22)	6.58	0.66	TSH, GH, ACTH, LH/FSH, AVP	APS, APP, ONH, ASP
10, M	2005	2972	SS, micropenis (1Y)	2.02 (5)		3.85	0.99	TSH, LH/FSH, PRL	Normal
11, M	2005	3168	Sc (31D)	0.37 (14)	0.62 (31)	3.29	0.53	TSH, ACTH	PH
12, M	2007	1786	Follow-up of HP (4M)	1.10[a] (6)	0.83 (28)	0.19	0.96	TSH, GH, ACTH, AVP	APS, APP, HP
13, M	2007	3122	Hypoglycemia (2D)	Not tested		3.34	0.88	TSH, GH, ACTH, LH/FSH	APS, EPP, PH
14, M	2007	3445	Sc (31D)	0.43 (6)	0.50 (31)	2.35	0.60	TSH, GH, ACTH, LH/FSH	EPP, PH

Pt. no., patient number; Wt, weight; Sc, screening; D, days old; M, months old; Y, years old; AVP, arginine vasopressin; PRL, prolactin; TSH, thyroid-stimulating hormone. APS, absent pituitary stalk; EPP, ectopic posterior pituitary; APP, absent posterior pituitary; ONH, optic nerve hypoplasia; ASP, absent septum pellucidum; PH, pituitary hypoplasia; HP, holoprosencephaly; SS, short stature; MR, magnetic resonance.
[a]This patient was born with low birth weight and hence this value was treated as unofficial.

Table 2 Characteristics of ten patients with CH-C categorized into isolated CH-C (group I; patients 15–22) and those with undetermined pituitary involvement (group U; patients 23 and 24).

	At birth			FT_4 values at Sc (ng/dl)		At first presentation			
Pt. no., sex	Year	Weight (g)	**Diagnostic symptom** (age)	1st sample (day)	2nd sample (day)	Serum TSH (μIU/ml)	Serum FT_4 (ng/dl)	**Basis for diagnosis of hypothyroidism**	**MR imaging of the CN**
15, M	2003	3370	Sc (14D)	0.14 (5)	0.48 (14)	2.86	0.45	Delayed TSH-R to TRH (5Y) Low FT_4 of 0.10 ng/dl (5Y)	Normal
16, M	2004	2770	SS (2Y)	1.79 (5)		2.20	0.55	Low FT_4 of 0.55 ng/dl (2Y)	Normal
17, M	2006	3450	Sc (15D)	0.60 (4)	0.47 (15)	2.79	1.01	Low FT_4 of 0.99 ng/dl on L-T_4 therapy (5Y) Requirement of high dose of L-T_4 (55 μg) to achieve NFR (5Y)	ND
18, M	2008	3060	Sc (24D)	0.68 (13)	0.68 (24)	1.86	0.94	Low TSH-R (6.90 μIU/ml) to TRH (1M)	Normal
19, M	2008	3868	Sc (12D)	0.43 (5)	0.66 (12)	3.02	0.72	Requirement of high dose of L-T_4 (55μg) to achieve NFR (11M)	ND
20, F	2007	3262	Sc (13D)	0.50 (5)	0.60 (13)	2.28	0.73	Low TSH-R (0.59 μIU/ml) to TRH (3M) Requirement of high dose of L-T_4 (55 μg) to achieve NFR (2Y)	ND
21, M	2008	3440	Sc (13D)	0.69 (4)	0.53 (13)	2.13	0.70	Low TSH-R (0.01 μIU/ml) to TRH (6M) Low FT_4 of 0.70 ng/dl (20D) and 1.10 ng/dl (6M)	PH
22, M	2008	3145	Sc (20D)	0.21 (5)	0.50 (20)	2.34	0.43	Low TSH-R to TRH (26D) Low FT_4 of 0.43 ng/dl (26D)	Normal
23, F	2007	668	Follow-up of low birth weight (1M)	0.27[a] (4)		Unknown	0.80	Low FT_4 of 0.70 ng/dl on L-T_4 therapy (2M)	Normal
24, M	2008	2542	Sc (15D)	0.57 (4)	0.57 (15)	1.24	0.98	Low FT_4 of 0.98 ng/dl (5M)	ND

Pt. no., patient number; D, days old; M, months old; Y, years old; L-T_4, levothyroxine; PH, pituitary hypoplasia; SS, short stature; Sc, Screening; TSH-R, TSH response; NFR, normal FT_4 range; MR, magnetic resonance; ND, not done.

[a]This patient had low birth weight, and the data obtained at 4 days of age were treated as unofficial. L-Thyroxine therapy was initiated before her first official sample was obtained.

CH-C in Kanagawa prefecture as 1 in 30 833 births (24/740 003).

Among the 24 patients, 14 patients (58%, ten males) were categorized into group M (Fig. 3). Group M ($n=14$) consisted of five patients with septo-optic dysplasia, five patients with pituitary hypoplasia, one with holoprosencephaly, and three with normal pituitary morphology. Eight other patients out of the 24 (33%) were considered to have isolated CH-C, without pituitary involvement, and they were hence categorized as group I (Fig. 3). Pituitary function in the remaining two patients could not be fully evaluated because of their younger age, and they were therefore categorized as group U (Fig. 3).

Twelve patients (50%) were identified as having CH-C solely via the newborn screening system in Kanagawa prefecture (Fig. 3). Of these, four patients belonged to group M, seven patients to group I, and one patient to group U. In addition, patient 6 in group M was clinically diagnosed with CH-C because this patient exhibited shock; however, the screening result was actually positive (low FT_4 levels), and hence, this was considered as a true-positive case of CH-C. Therefore, the total number of true-positive CH-C cases was 13. In contrast, nine other patients out of 24 (38%, eight patients in group M and one in group I) had normal screening results and were revealed to have CH-C through the evaluation of clinical symptoms such as shock and/or hypoglycemia during the neonatal period ($n=2$), short stature ($n=4$), and other features ($n=3$). These nine patients were considered to be false negatives. The remaining two patients (one in group M and one in group U, depicted as '?' in Fig. 3) were already on L-T_4 treatment before screening, and hence, they were excluded from the judgment as to whether the screening results were positive or negative as they had already been diagnosed with CH-C. Patients in group I were significantly identified more frequently through the screening program than those in group M: 88% (7/8) vs 29% (4/14), $P<0.01$.

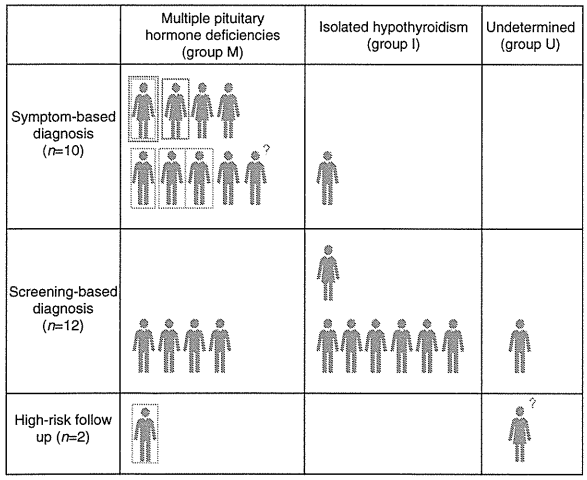

Figure 3 Summary of the 24 patients with CH-C, categorized by presence/absence of other pituitary hormone deficiencies and diagnostic symptoms. The red and blue figures indicate female and male patients respectively. Figures within a single-line box indicate CH-C patients who could have been identified as having CH-C by screening if the FT_4 cutoff values were 0.9 ng/dl. The figure within a double-line box indicates the patient diagnosed with septo-optic dysplasia presenting with shock, who had FT_4 levels <0.7 ng/dl according to the results of the filter paper assay. $^?FT_4$ data with the filter paper assay were not available for two patients; L-thyroxine treatment was initiated in one male patient at 2 days of age. One female patient had low birth weight, and the data obtained at 4 days of age were treated as unofficial. L-Thyroxine therapy was initiated before the first official sample was obtained from this patient.

Out of the 24 CH-C patients, for 22 patients the filter paper assay for FT_4 showed clear positive or negative results during screening (Fig. 4). The remaining two patients had been started on L-T_4 therapy before screening. Because no blood samples were collected from any patient between 8 and 11 days of age, FT_4 measurements were arbitrarily divided into those obtained on or before 10 days of age (FT_4 before 10D, $n=18$, collected from 18 patients) and those obtained on or after 11 days of age (FT_4 after 11D, $n=16$, collected from 14 patients). Overall, the FT_4 level before 10D was 0.82 ± 0.56 ng/dl (median, 5 days of age; range, 4–7 days), whereas the FT_4 level after 11D was 0.57 ± 0.13 ng/dl (median, 17.5 days; range, 12–31 days; Fig. 4). In addition, when we analyzed the data exclusively obtained from patients whose FT_4 levels had been determined twice ($n=10$), no significant difference was observed between FT_4 before 10D (0.46 ± 0.05 ng/dl) and FT_4 after 11D (0.52 ± 0.02 ng/dl). Thus, FT_4 values in CH-C patients appeared to be stable during the neonatal period. A comparison of FT_4 levels in group M ($n=17$) with those in group I ($n=15$) also did not show a statistically significant difference (group M, 0.81 ± 0.49 ng/dl; group I, 0.60 ± 0.37 ng/dl), indicating that the severity of hypothyroidism did not differ significantly between these two groups, differentiated by pituitary involvement.

Evaluation of the performance of the screening system is depicted in Table 3. Our screening system yielded 13 true positives and nine false negatives, so that the sensitivity of detection of a true positive was calculated to be 59.1%. Specificity and PPVs were calculated to be 99.99 and 11.5% respectively. A total of 740 003 newborns were screened during the study period and 113 newborns were sent for thorough evaluation based on two consecutive FT_4 measurements. The cutoff level used was 0.7 ng/dl serum (9.0 pmol/l). In the next step, we simulated the performance of the screening system with higher cutoff values. As depicted in Fig. 4, FT_4 levels for nine patients who were not identified in the screening ranged from 0.81 to 2.02 ng/dl (median, 0.9 ng/dl), which was substantially lower than the reference range of

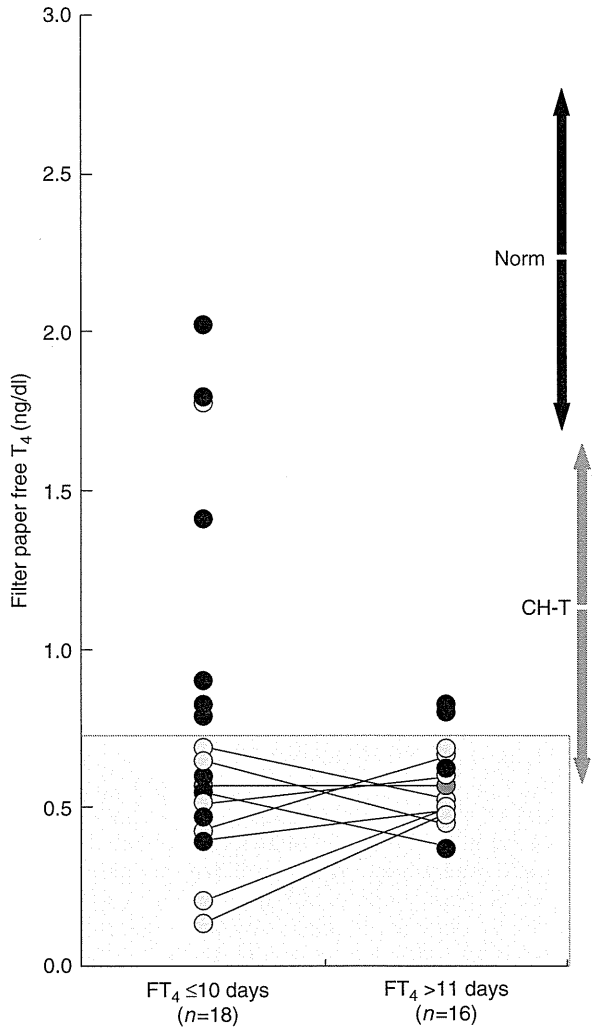

Figure 4 Distribution of FT_4 values from the filter paper assay for 22 patients with CH-C. FT_4 measurements obtained before 10 days of age (FT_4 before 10D) and those obtained after 11 days (FT_4 after 11D) did not differ significantly. The black circles indicate FT_4 obtained from patients in group M (with multiple pituitary hormone deficiencies), the yellow circles indicate FT_4 obtained from patients in group I (isolated hypothyroidism), and the red circles indicate FT_4 obtained from patient 24. The shaded area indicates FT_4 values below the cutoff of 0.7 ng/dl. Determinants from the same individuals are connected by solid lines. The black arrow (norm) indicates the mean ± 1 S.D. (2.22 ± 0.58 ng/dl) of FT_4 values from the filter paper assay conducted on 67 933 normal newborns. The blue arrow (CH-T) indicates the mean ± 1 S.D. (1.08 ± 0.54 ng/dl) of FT_4 values from filter paper assay on 61 patients diagnosed with CH-T.

1.64–2.80 ng/dl (21.1–36.0 pmol/l; data obtained from the 67 933 normal newborns). If the cutoff value is raised to 0.9 ng/dl serum (11.6 pmol/l), then an additional five patients would have been found to be positive by the screening, and the estimated sensitivity would be increased by 81.8%.

Discussion

In Japan, two types of ELISA-based kits are available for measuring FT_4 levels in dried blood samples on filter paper; one developed by Siemens Healthcare Diagnostics K.K and another by Eiken Chemical Co. Ltd. Because TSH and FT_4 can be measured with a common detection module, additional costs for FT_4 measurements are only those incurred for reagents: 465 yen for TSH alone vs 705 yen for TSH and FT_4 determination per newborn examined. Most of the screening centers adopt a primary TSH and backup FT_4 system: the filter paper method is used for measuring TSH in all newborns, while it is used for measuring FT_4 only in those with high TSH values for confirmation of possible hypothyroidism (11, 12). To detect CH-C, certain areas, including Kanagawa prefecture and Sapporo city, have adopted a combined primary TSH–FT_4 screening system (7, 9). After the report from Sapporo city (7), this report is the second audit of this CH-C newborn screening system, conducted on a larger population and for a longer study period. We also tried to trace CH-C patients not identified by neonatal screening (false-negative cases).

The ELISA-based filter paper FT_4 kits are almost exclusively used in Japan. One may argue against its accuracy in determining FT_4 levels, considering that some TBG-deficient patients were falsely detected to have low FT_4 levels and that the equilibrium dialysis method is the gold standard (15, 16). However, it has been difficult to introduce the equilibrium dialysis method in newborn screening because it requires a larger volume serum sample and longer measurement times. On the other hand, to use the FT_4 index instead of FT_4, tri-iodothyronine (T_3) uptake must also be measured, which increases cost. FT_4 determined by ELISA on filter paper blood samples seems to correctly reflect FT_4 status in newborns because most (88%) FT_4 values in CH-C patients were more than 2 S.D. below the mean of normal newborns and because FT_4 levels in CH-T were distributed in a substantially low range (0.04–2.32 ng/dl; Fig. 4). Moreover, Fig. 4 shows that the FT_4 levels measured using the filter paper method may be consistent even at lower concentrations of FT_4. Thus, we believe that although FT_4 levels determined using the filter paper samples may not be identical to those measured by the equilibrium dialysis method, the assay is a promising, practical alternative for use in CH-C screening. Because combined TSH-T_4 is recommended as the ideal strategy for detecting both CH-C and CH-T by the American Thyroid Association and Pediatric Endocrine Societies in the US and Europe (2), we think it is justified to continue implementation of our combined TSH–FT_4 system as a new version of the TSH–T_4 system.

From our survey, the incidence of CH-C was calculated as 1 in 30 833 live births, while that of CH-T was 1 in 3472 live births. Thus, the CH-T/CH-C ratio in this study was 8.9, which is close to the ratio 8.4 reported from

Table 3 Simulation of sensitivity and PPV of the screening system for CH-C based on varying FT_4 cutoff values.

FT_4 cutoff[a] (ng/dl (pmol/l) serum)	Newborns screened	Newborns asked for second sample (% of the total)	Newborns sent for evaluation (% of the total)	CH-C patients identified by screening (true positives)	CH-C patients missed by screening (false negatives)	Sensitivity (%)	PPV (%)
Kanagawa prefecture results (1999–2008)							
0.7 (9.0)	740 003	1220 (0.16)	113 (0.015)	13[b]	9	59.1	11.5
Simulation of data from Kanagawa prefecture							
0.9 (11.6)	740 003	3735 (0.50)	Unknown	18[c]	4	81.8	Unknown
1.0 (12.9)	740 003	6656 (0.90)	192[d]	18[c]	4	81.8	9.4
Sapporo city results (2004–2008, reference (7))							
1.0 (12.9)	83 232	629 (0.76)	22 (0.026)	6	Unknown	Unknown	27.3

[a]FT_4 cutoff of 0.7 ng/dl (9.0 pmol/l) was used during the survey.
[b]Twelve patients identified entirely via screening (screening-based diagnosis in Fig. 3) and one who developed shock and had FT_4 below 0.7 ng/dl at screening (patient 6 in Table 1, who is indicated by the figure surrounded by double lines in the symptom-based diagnosis in Fig. 3).
[c]Twelve patients identified entirely via screening (screening-based diagnosis in Fig. 3) and six who were diagnosed clinically but had FT_4 below 0.9 ng/dl (figures surrounded by a single line in the symptom-based diagnosis in Fig. 3).
[d]This figure was deduced from the incidence of 0.026% in Sapporo city (reference (7)).

The Netherlands (6). Although we previously reported a much lower CH-C incidence (1 in 160 516 births) (9), that survey was based on only the cases detected through screening. The incidence rate of 1 in 30 833 reported here is likely to be underestimated because this study was based on a questionnaire survey and false-negative cases may not have been recorded. Indeed, we could not obtain follow up data on 11 cases identified in the preliminary survey, as well as on eight patients with positive screening results. In addition, because correct diagnosis of CH-C is difficult (17, 18), especially in those with isolated hypothyroidism, some cases may have been overlooked. Moreover, as shown in Fig. 4, the mean values and range of FT_4 in CH-C patients were lower than those in CH-T patients, suggesting that milder forms of CH-C may escape detection.

A remarkable finding in this study is that isolated hypothyroidism (group I) was detected in one-third of the total CH-C population. Previous studies have found that 78% (5) to 98% (8) of CH-C patients had multiple pituitary hormone defects such as septo-optic dysplasia. There are some explanations for this discrepancy. First, isolated CH-C patients present less prominent symptoms than those with multiple pituitary hormone deficiencies (19, 20, 21, 22) and hence may be missed in the absence of screening. Indeed, all but one patient in group I was identified through the newborn screening. A Dutch screening system with TSH, T_4, and TBG determination (5) reported a prevalence rate of 22% of isolated CH-C, which is closer to our findings. Secondly, ethnic differences may be a factor: in Sapporo city, two of six CH-C patients were reported to demonstrate isolated hypothyroidism (7). Thirdly, some patients may not have been correctly diagnosed: a patient (patient 21 in Table 2) with pituitary hypoplasia is likely to have other hormone deficiencies. Finally, transient hypothyroidism may not be definitively ruled out, especially in younger patients. However, the authors are aware of a patient in group I (patient 15) who demonstrated severe hypothyroidism when L-T_4 therapy was tentatively interrupted. Reevaluation of all other patients in group I will determine the true incidence of isolated hypothyroidism.

Our current system yielded a sensitivity of 59.1% and PPV of 11.0% in detecting CH-C. In fact, 12 patients were diagnosed with CH-C entirely on the basis of low FT_4 levels at newborn screening. Above all, the presence of four patients in group M, who were overlooked clinically but in whom low FT_4 levels were detected at screening, underscores the usefulness of our combined primary TSH–FT_4 system. The sensitivity of 59.1% seems superior to the reported sensitivity of 19.0% in the state of Indiana, USA, where T_4 measurement was used (8). On the other hand, a study from The Netherlands reported the sensitivity to be 71.4% (6). Because our study relied on responses to a questionnaire, the actual sensitivity of our screening system may be lower: physicians who did not respond may have

CH-C patients who were missed in the screening, some unrecognized cases with isolated hypothyroidism may be present, and early death of patients with multiple pituitary hormone deficiencies may have been ignored. Thus, we cannot directly compare the performance of our system to that used in The Netherlands.

Setting a higher cutoff for FT_4 has both advantages and disadvantages. At a cutoff of 0.9 ng/dl instead of 0.7 ng/dl, the estimated sensitivity rises to 81.8%; it may increase three times more considering the requests for a second filter paper test (retesting ratio, 0.50%). In Sapporo city, the FT_4 cutoff has been set to 1.0 ng/dl (7); six CH-C patients were identified through the screening over 4 years, and the prevalence of CH-C was reported to be 1 in 13 872 live births (Table 3). If a cutoff of 0.7 ng/dl were to be applied to their cohort, only one of six CH-C patients would have been detected by screening. Thus, resetting the cutoff value to 0.9 ng/dl (or higher) may be necessary. This level is in accordance with the FT_4 cutoff 0.93 ng/dl used in The Netherlands for the diagnosis of CH-C (5) and is ~ -2 S.D. of both the reported cord blood values (23) and our FT_4 values (Fig. 3) for normal newborns.

Adequacy of the retesting ratio depends on many factors including population size, system performance parameters such as sensitivity and PPV, and local economic conditions. The retesting ratio was as high as 0.76% in Sapporo city, due to a higher cutoff value and inclusion of low-birth weight newborns. This ratio may be acceptable in a smaller city but may not be suitable for Kanagawa prefecture. The estimated retesting ratio of 0.50% (3735 samples during 10 years) resulting from a cutoff of 0.9 ng/dl may be more acceptable than a ratio of 0.76%. A comparative figure for retesting for congenital adrenal hyperplasia in Kanagawa prefecture is 0.3%. Because FT_4 determinants did not change significantly according to collection dates (Fig. 4), as shown also in normal newborns (13), differential cutoff values according to the sampling dates are not expected to reduce the retesting ratio.

Another problem is the introduction of an immediate evaluation system to facilitate early treatment of CH-C patients, especially for those with multiple pituitary hormone deficiencies. As stated in the Subjects and methods section, unless two consecutive tests reveal low FT_4 values, newborns will not be subject to a thorough evaluation in our system. The aim of performing a second sampling is to exclude false positives. Indeed, during the study period, second samples were requested for 1220 newborns, but only 113 of these newborns were sent for thorough evaluation and 1107 false-positive cases were eliminated (Table 3). Even with a cutoff of 1.0 ng/dl, the number of newborns sent for evaluation will increase minimally (79 additional cases across 10 years). We retrospectively analyzed the impact of introduction of an immediate evaluation system in which newborns with FT_4 lower than 0.5 ng/dl (6.4 pmol/l) will be immediately evaluated. Seven CH-C patients, including three patients in group M, would have been diagnosed without delay. However, according to our simulation, this strategy will create a false-positive number of more than 200 over 10 years, with a PPV of 2.8%. The question of whether this figure is reasonable is beyond the scope of our study. Nevertheless, we plan to improve our screening strategy by considering scientific, economical, ethical, and political issues.

In conclusion, measurement of FT_4 in dried blood spots on filter paper is suitable for newborn screening for CH-C; moreover, the combined primary TSH–FT_4 system applied in Kanagawa prefecture identified a significant number of CH-C patients before they manifested clinical symptoms. The survey identified 24 CH-C patients, 14 of whom had multiple pituitary hormone deficiencies, yielding an incidence rate of CH-C of 1 in 30 833 live births. Screening sensitivity was calculated to be 59.1%, based on 13 true-positive cases and nine false-negative cases, with a cutoff of 0.7 ng/dl of FT_4. A more appropriate (higher) FT_4 cutoff value and proper implementation of the screening would facilitate early detection of CH-C cases.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

M Adachi, Y Yamagami, and F Hirahara conceptualized and designed the study. M Adachi and A Soneda contributed to the data collection, analysis, and writing of the manuscript. Y Asakura and K Muroya contributed to preparation of the manuscript by critically analyzing it.

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References

1 Büyükgebiz A. Newborn screening for congenital hypothyroidism. *Journal of Pediatric Endocrinology & Metabolism* 2006 **19** 1291–1298. (doi:10.1515/JPEM.2006.19.11.1291)

2 American Academy of Pediatrics, Rose SR; Section on Endocrinology and Committee on Genetics, American Thyroid Association, Brown RS; Public Health Committee, Lawson Wilkins Pediatric Endocrine Society, Foley T, Kaplowitz PB, Kaye CI, Sundararajan S & Varma SK. Update of newborn screening and therapy for congenital hypothyroidism. *Pediatrics* 2006 **117** 2290–2303. (doi:10.1542/peds.2006-0915)

3 Hanna CE, Krainz PL, Skeels MR, Miyahira RS, Sesser DE & LaFranchi SH. Detection of congenital hypopituitary hypothyroidism: ten-year experience in the Northwest Regional Screening Program. *Journal of Pediatrics* 1986 **109** 959–964. (doi:10.1016/S0022-3476(86)80276-1)
4 Hunter MK, Mandel SH, Sesser DE, Miyabira RS, Rien L, Skeels MR & LaFranchi SH. Follow-up of newborns with low thyroxine and nonelevated thyroid-stimulating hormone-screening concentrations: results of the 20-year experience in the Northwest Regional Newborn Screening Program. *Journal of Pediatrics* 1998 **132** 70–74. (doi:10.1016/S0022-3476(98)70487-1)
5 van Tijn DA, de Vijlder JJ, Verbeeten B Jr, Verkerk PH & Vulsma T. Neonatal detection of congenital hypothyroidism of central origin. *Journal of Clinical Endocrinology and Metabolism* 2005 **90** 3350–3359. (doi:10.1210/jc.2004-2444)
6 Kempers MJ, Lanting CI, van Heijst AF, van Trotsenburg AS, Wiedijk BM, de Vijlder JJ & Vulsma T. Neonatal screening for congenital hypothyroidism based on thyroxine, thyrotropin, and thyroxine-binding globulin measurement: potentials and pitfalls. *Journal of Clinical Endocrinology and Metabolism* 2006 **91** 3370–3376. (doi:10.1210/jc.2006-0058)
7 Fujiwara F, Fujikura K, Okuhara K, Tsubaki J, Fukushi M, Fujita K, Fujieda K & Tajima T. Central congenital hypothyroidism detected by neonatal screening in Sapporo, Japan (2000–2004): it's prevalence and clinical characteristics. *Clinical Pediatric Endocrinology* 2008 **17** 65–69. (doi:10.1297/cpe.17.65)
8 Nebesio TD, McKenna MP, Nabhan ZM & Eugster EA. Newborn screening results in children with central hypothyroidism. *Journal of Pediatrics* 2010 **156** 990–993. (doi:10.1016/j.jpeds.2009.12.011)
9 Asakura Y, Tachibana K, Adachi M, Suwa S & Yamagami Y. Hypothalamo-pituitary hypothyroidism detected by neonatal screening for congenital hypothyroidism using measurement of thyroid-stimulating hormone and thyroxine. *Acta Paediatrica* 2002 **91** 172–177. (doi:10.1080/080352502317285171)
10 LaFranchi S. Congenital hypothyroidism: etiologies, diagnosis, and management. *Thyroid* 1999 **9** 735–740. (doi:10.1089/thy.1999.9.735)
11 Gu YH, Kato T, Harada S, Inomata H, Saito T & Aoki K. Seasonality in the incidence of congenital hypothyroidism in Japan: gender-specific patterns and correlation with temperature. *Thyroid* 2007 **17** 869–874. (doi:10.1089/thy.2006.0317)
12 Nagasaki K, Asami T, Ogawa Y, Kikuchi T & Uchiyama M. A study of the etiology of congenital hypothyroidism in the Niigata prefecture of Japan in patients born between 1989 and 2005 and evaluated at ages 5–19. *Thyroid* 2011 **21** 361–365. (doi:10.1089/thy.2010.0005)
13 Gruñeiro-Papendieck L, Prieto L, Chiesa A, Bengolea S, Bossi G & Bergadá C. Usefulness of thyroxine and free thyroxine filter paper measurements in neonatal screening for congenital hypothyroidism of preterm babies. *Journal of Medical Screening* 2000 **7** 78–81. (doi:10.1136/jms.7.2.78)
14 Fukushi M, Mashimo Y, Mizushima Y, Arai O, Kikuchi Y, Takasugi N, Fujieda K & Matsuura N. Examination of enzaplate, N-FT_4 and its application for neonatal thyroid screening. *Clinical Endocrinology* 1990 **38** 1039–1042.
15 Nelson JC & Tomei RT. Direct determination of free thyroxin in undiluted serum by equilibrium dialysis/radioimmunoassay. *Clinical Chemistry* 1988 **34** 1737–1744.
16 Deming DD, Rabin CW, Hopper AO, Peverini RL, Vyhmeister NR & Nelson JC. Direct equilibrium dialysis compared with two non-dialysis free T4 methods in premature infants. *Journal of Pediatrics* 2007 **151** 404–408. (doi:10.1016/j.jpeds.2007.03.046)
17 Mehta A, Hindmarsh PC, Stanhope RG, Brain CE, Preece MA & Dattani MT. Is the thyrotropin-releasing hormone test necessary in the diagnosis of central hypothyroidism in children. *Journal of Clinical Endocrinology and Metabolism* 2003 **88** 5696–5703. (doi:10.1210/jc.2003-030943)
18 Faglia G, Bitensky L, Pinchera A, Ferrari C, Paracchi A, Beck-Peccoz P, Ambrosi B & Spada A. Thyrotropin secretion in patients with central hypothyroidism: evidence for reduced biological activity of immunoreactive thyrotropin. *Journal of Clinical Endocrinology and Metabolism* 1979 **48** 989–998. (doi:10.1210/jcem-48-6-989)
19 Pittman JA Jr, Haigler ED Jr, Hershman JM & Pittman CS. Hypothalamic hypothyroidism. *New England Journal of Medicine* 1971 **285** 844–845. (doi:10.1056/NEJM197110072851508)
20 Niimi H, Inomata H, Sasaki N & Nakajima H. Congenital isolated thyrotrophin releasing hormone deficiency. *Archives of Disease in Childhood* 1982 **57** 877–878. (doi:10.1136/adc.57.11.877)
21 Collu R, Tang J, Castagné J, Lagacé G, Masson N, Huot C, Deal C, Delvin E, Faccenda E, Eidne KA & Van Vliet G. A novel mechanism for isolated central hypothyroidism: inactivating mutations in the thyrotropin-releasing hormone receptor gene. *Journal of Clinical Endocrinology and Metabolism* 1997 **82** 1561–1565. (doi:10.1210/jc.82.5.1561)
22 Bonomi M, Busnelli M, Beck-Peccoz P, Costanzo D, Antonica F, Dolci C, Pilotta A, Buzi F & Persani L. A family with complete resistance to thyrotropin-releasing hormone. *New England Journal of Medicine* 2009 **360** 731–734. (doi:10.1056/NEJMc0808557)
23 Williams FL, Simpson J, Delahunty C, Ogston SA, Bongers-Schokking JJ, Murphy N, van Toor H, Wu SY, Visser TJ, Hume R & Collaboration from the Scottish Preterm Thyroid Group . Developmental trends in cord and postpartum serum thyroid hormones in preterm infants. *Journal of Clinical Endocrinology and Metabolism* 2004 **89** 5314–5320. (doi:10.1210/jc.2004-0869)

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CLINICAL STUDY

Functional characterization of four novel *PAX8* mutations causing congenital hypothyroidism: new evidence for haploinsufficiency as a disease mechanism

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Abstract

Background: Individuals carrying a heterozygous inactivating *PAX8* mutation are affected by congenital hypothyroidism (CH), although heterozygous *Pax8* knockout mice are not. It has remained unclear whether CH in *PAX8* mutation carriers is caused by haploinsufficiency or a dominant negative mechanism.

Objective: To report clinical and molecular findings of four novel *PAX8* mutations, including one early-truncating frameshift mutation.

Subjects and methods: Four probands were CH patients. Two had family history of congenital or childhood hypothyroidism. Three probands were diagnosed in the frame of newborn screening for CH, while one had a negative result in screening but was diagnosed subsequently. Three had thyroid hypoplasia and one had a slightly small thyroid with low echogenicity. For these probands and their family members, we sequenced *PAX8* using a standard PCR-based method. Pathogenicity of identified mutations was verified *in vitro*.

Results: We found four novel heterozygous *PAX8* mutations in the four probands: L16P, F20S, D46SfsX24, and R133Q. Family studies showed four additional mutation carriers, who were confirmed to have high serum TSH levels. Expression experiments revealed that three mutations (L16P, F20S, and R133Q) had defects in target DNA binding, while D46fs had protein instability that was rescued by the proteasome inhibitor MG132. All four mutations had reduced transactivation on the thyroglobulin promoter, supporting that they were inactivating mutations.

Conclusion: D46fs is the first *PAX8* mutation with confirmed protein instability. Our clinical and *in vitro* findings together suggest that pure *PAX8* haploinsufficiency can cause CH in humans.

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Introduction

PAX8, a member of the Pax gene family, plays pivotal roles in thyroid development and physiology. PAX8 is expressed in the developing thyroid to adulthood (1). PAX8 directly regulates transcription of thyroid-specific genes, such as thyroglobulin (Tg), in cultured cell lines (2). *Pax8* knockout mice have thyroid aplasia due to defective proliferation and survival of thyroid precursor cells (3). In humans, a heterozygous *PAX8* mutation causes congenital hypothyroidism (CH). To date, 32 mutation carriers belonging to 13 families have been described (4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14). Clinical phenotypes of mutation carriers are variable, ranging from overt CH with severe thyroid hypoplasia to subclinical CH with a morphologically normal gland. Detected mutations include ten amino acid-altering mutations in the DNA-binding paired domain (R31C, R31H, Q40P, S48F, R52P, S54G, H55Q, C57Y, L62R, and K80_A84dup) and two protein-truncating mutations (R108X and T277X). No consistent genotype–phenotype correlation has been suggested.

Several *PAX* genes other than *PAX8* have been implicated in Mendelian disorders, including *PAX2* (papillorenal syndrome; OMIM*167409), *PAX3* (Waardenburg syndrome; OMIM *606597), *PAX6* (aniridia; OMIM *607108), and *PAX9* (tooth agenesis; OMIM *167416). All four genes, when mutated, are assumed to cause a human disease via haploinsufficiency because both entire gene deletion(s) and nucleotide-level mutation(s) with early protein truncation produce disease phenotypes (Supplementary Table 1, see section on supplementary data given at the end of this article). This assumption is also supported by observations of

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mutant mice with inactivating *Pax* allele(s), showing the gene dosage effect (e.g. disease phenotype seen in heterozygotes; Supplementary Table 1). Contrastingly, the mechanism linking heterozygous *PAX8* mutations and CH in humans has remained obscure. To date, neither entire *PAX8* deletion nor early truncation mutation has been reported. Moreover, heterozygous *Pax8*-knockout mice are not affected by CH (15). Based on the phenotypic difference between mutation-carrying human patients and mice, a dominant negative effect in the former has been proposed (7), although most previous *in vitro* studies have failed to recapitulate the effect.

Here, we report the identification and functional characterization of four novel CH-associated *PAX8* mutations. Among the four mutations, one was a frameshift mutation (D46fs) causing protein instability *in vitro*. Our clinical and molecular findings about the first experimentally confirmed null *PAX8* mutation provides new evidence, indicating that *PAX8* haploinsufficiency can cause CH in humans.

Materials and methods

Mutation detection

This study was approved by the Institutional Review Board of Keio University School of Medicine. We obtained written informed consent for molecular studies from the study subjects or his/her parents. Leukocytic DNAs were extracted from the four probands and their family members with the Gentra Puregene Blood Kit (Qiagen). Coding exons and flanking introns of *PAX8* (transcript variant PAX8A; GenBank NM_003466.3) were analyzed by standard PCR-based sequencing as described previously (12). Detected mutations were tested in 100 control Japanese individuals.

Three-dimensional modeling

Three-dimensional structures of three missense mutants (L16P, F20S, and R133Q) were modeled with 3D-JIGSAW (http://bmm.cancerresearchuk.org/~3djigsaw/). The structure data of PAX6–DNA complex (protein data bank ID 6PAX; http://www.rcsb.org/pdb) were used as a template. The pictures of the modeled structures were produced with PyMOL (http://www.pymol.org).

Plasmids, cell culture, and transfection

Vectors encoding human PAX8 cDNA (untagged, myc tagged, or enhanced green fluorescent protein (EGFP) tagged) have been described previously (12). The four mutations (L16P, F20S, D46fs, and R133Q) were introduced into these vectors by site-directed mutagenesis (QuikChange XL Site-Directed Mutagenesis Kit; Agilent Technologies, Santa Clara, CA, USA). All final constructs were verified by direct sequencing. HeLa cells were maintained in DMEM supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, and 10% fetal bovine serum. For functional assays, cells were transfected with DNA using Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocol.

Western blotting

Cells transfected with each of myc-tagged PAX8 constructs (wild type (WT) or mutant) or the empty vector were harvested at 24 h after transfection. Crude cell lysate was obtained with the M-PER protein extraction reagent (Pierce, Rockford, IL, USA). Samples containing 20 μg protein were separated on 10% SDS–PAGE, and western blotting was performed with a mouse anti-myc MAB (Life Technologies) and a HRP-conjugated rabbit anti-mouse IgG polyclonal antibody (Sigma–Aldrich) as a second antibody. Bound antibody was revealed with a chemiluminescence kit (GE Healthcare, Buckinghamshire, UK).

Cells transfected with the myc-tagged D46fs mutant or the empty vector were treated with dimethyl sulfoxide (DMSO) or DMSO containing 1 μM MG132 (Sigma–Aldrich) for an additional 12 h. Western blotting analyses were performed as described earlier.

Visualization of subcellular localization

Cells grown on sterile glass coverslips were cotransfected with each PAX8–EGFP fusion construct (WT or mutant) and the vector encoding red fluorescent protein-tagged thyroid transcription factor-1 (TTF1). Twenty-four hours after transfection, cells were fixed in 2% formaldehyde/PBS at room temperature for 10 min. Then, coverslips were mounted with Vectashield Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA) and were observed under a TCS-SP5 confocal microscope (Leica Microsystems, Mannheim, Germany).

Electrophoretic mobility shift assay

The two band shift probes, oligo-CT (TGA TGC CCA CTC AAG CTT AGA CAG) and oligo-C (CAC TGC CCA GTC AAG TGT TCT TGA), were prepared by annealing of 3′-biotin-labeled complementary oligonucleotides (purchased from BEX Co., Ltd, Tokyo, Japan). Five micrograms of nuclear protein extraction (prepared with the NE-PER nuclear extraction reagent (Pierce)) were incubated at room temperature in 20 μl binding reaction mixture containing 20 fmol probe, 50 mM KCl, 5 mM $MgCl_2$, 2.5% glycerol, 0.05% NP-40, and 1 μg poly (dI-dC) for 20 min. For competition experiments, a large excess (200×) of unlabeled competitor oligonucleotides was included in the binding reactions. The protein–DNA complexes were subject to gel