adipose tissue of obese women, resulting in elevated peripheral aromatization of androgens and increased circulating estrogen levels (Geisler *et al.* 2007, Maccio *et al.* 2009, Hursting 2011). This may underlie the association between obesity and increased risk of breast cancer in postmenopausal women (Brown *et al.* 2009, Brown & Simpson 2010).

Aromatase inhibitors are a first-line therapy for estrogen receptor-positive breast cancer in postmenopausal women. However, due to systemic suppression of estrogen biosynthesis, treatment with aromatase inhibitors often leads to side effects associated with estrogen depletion, including arthralgia, bone loss, and bone fracture, as well as possible cardiovascular and neurocognitive defects (Thurlimann *et al.* 2005, Buzdar *et al.* 2006, Coombes *et al.* 2007). As a result of these adverse effects and also the high costs of aromatase inhibitors, 23–30% of patients could not complete aromatase inhibitor therapy (Hershman *et al.* 2010, Sedjo & Devine 2011). With this high rate of nonadherence in patients with life-threatening cancer, it is reasonable to believe that patient acceptance of aromatase inhibitors as a breast cancer chemopreventive option could be limited. Therefore, it is imperative to develop new agents with lower toxicities and lower costs for breast cancer prevention.

Low serum and toenail selenium levels (a measure of long-term selenium intake) are associated with an increased risk of breast cancer (Charalabopoulos *et al.* 2006, Suzana *et al.* 2009, Kotsopoulos *et al.* 2010). Selenium supplementation has been demonstrated by numerous preclinical studies to effectively inhibit the development of breast cancer (Ip 1998, Ip *et al.* 2002), while having a low toxicity profile (Reid *et al.* 2004). The anticancer efficacy depends on the form and dosage of selenium administered (Ip 1998, Ip *et al.* 2000, Li *et al.* 2004, 2008, Wang *et al.* 2009). Methylseleninic acid (MSA) is a potent second-generation selenium compound. It has very different biological and pharmacological activity than selenomethionine, the form of selenium used in the selenium and vitamin E chemoprevention trial (Ip 1998, Ip *et al.* 2000, Li *et al.* 2008, Lippman *et al.* 2009, Ohta *et al.* 2009, Wang *et al.* 2009). In the present study, we characterized the effect of MSA on estrogen biosynthesis that has never been investigated before. We focused on the effect on promoters PI.4- and PII-driven aromatase expression because of the important role of these two promoters in regulating estrogen level in obese postmenopausal women. The long-term objective of the present study is to develop MSA as a low-cost, low-toxicity breast cancer chemopreventive agent for obese postmenopausal women.

Materials and Methods

Cell culture and reagents

The KGN human ovarian granulosa tumor cell line was established from a postmenopausal patient with invasive ovarian granulosa cell carcinoma (Nishi *et al.* 2001). KGN cells are undifferentiated, and maintain physiological characteristics of ovarian cells, including the expression of functional FSH receptor, relatively high aromatase activity, and the expression of estrogen receptor-β as the predominant isoform of estrogen receptor (Nishi *et al.* 2001, Chu *et al.* 2004). The cells were regularly cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The human preadipocyte cell strain, SGBS, was derived from an adipose depot of an infant with Simpson–Golabi–Behmel syndrome (Wabitsch *et al.* 2001). SGBS cells were routinely cultured in growth medium comprising DMEM/F12 medium supplemented with 10% FBS, 1% penicillin/streptomycin, 33 μM biotin, and 17 μM pantothenic acid. All the SGBS cells used in this study were in passage 30 to passage 35. The cells were switched to hormone-deprived medium (phenol red-free medium containing charcoal-stripped-FBS) for analysis of aromatase activity, protein, and mRNA. MSA was obtained from PharmaSe (Lubbock, TX, USA). ^{3}H-Androst-4-ene-3,17-dione was from PerkinElmer (Waltham, MA, USA). Dexamethasone (Dex), forskolin (FSK), and other reagents were purchased from Sigma–Aldrich.

Establishment of aromatase-overexpressing MCF-7arom cells

The aromatase cDNA with the 129 bp 5′-UTR sequence was PCR amplified from the CYP19A1-coding plasmid (SC107980, OriGene, Rockville, MS, USA) and subcloned into pcDNA3.1/Zeo (+) between the HindIII and XbaI sites. The resulting pcDNA3.1-aromatase construct was transfected to MCF-7 cells and selected with 100 μg/ml Zeocin for 3 months to generate stable aromatase-overexpressing MCF-7 cells, MCF-7arom. MCF-7arom cells were regularly cultured in DMEM medium containing 5% FBS, 1% penicillin/streptomycin, and 50 μg/ml Zeocin.

Aromatase activity assay

Aromatase activity was determined by using the tritiated water release assay that measures the amount of $^{3}H_2O$ formed during the conversion of ^{3}H-androstenedione to estrone by aromatase (Silva *et al.* 1989). SGBS cells were seeded to six-well plates (2×10^{4} cells/well) in hormone-deprived medium and allowed to attach overnight. The cells were treated with 250 nM Dex in the presence or absence of MSA for 6 or 16 h, and then washed twice with PBS before being incubated in fresh hormone-deprived medium containing 6 nM ^{3}H-androst-4-ene-3,17-dione for an additional 4 h. Following incubation, the medium was removed and extracted with two volumes of chloroform. The samples were then centrifuged at 2000 *g* for 10 min, and the aqueous upper layer was mixed with 2% charcoal followed by an additional centrifugation at 12 000 *g* for 10 min to remove any trace amount of unreacted substrate. A 500 μl aliquot of the supernatant for each sample was subsequently subjected to liquid scintillation counting.

For KGN cells, the aromatase activity assay was performed the same way as for SGBS cells except that 25 μM FSK were

used to replace Dex to induce aromatase activity. For determining the direct effect of MSA on aromatase enzymatic activity in SGBS and KGN cells, MSA was not added to the Dex- or FSK-containing medium, but was present during the last 4 h of incubation together with ^{3}H-androst-4-ene-3,17-dione.

Western blot analysis

KGN cells were seeded to hormone-deprived medium and cultured overnight. The cells were treated with FSK in the presence or absence of MSA for 6 or 16 h. Following treatment, the cells were washed twice with ice-cold PBS, and scraped in lysis buffer (Cell Signaling, Danvers, MA, USA). SDS–PAGE and western blotting procedures were done as described before (Liu *et al.* 2010). The mouse aromatase monoclonal antibody, 677/H7, was developed as described (Sasano *et al.* 2005), and the GAPDH antibody was obtained from Millipore (Billerica, MA, USA).

Aromatase total mRNA quantification

SGBS and KGN cells were seeded to hormone-deprived medium and cultured overnight before treatment. The cells were treated with MSA for 3 h. RNA extraction and real-time RT-PCR procedures were done as previously described (Dong *et al.* 2004). The primer–probe sets for aromatase (Hs00903409-m1) and β-actin (Hs99999903-m1) were from Applied Biosystems (Carlsbad, CA, USA).

Promoter-specific aromatase mRNA quantification

Promoter-specific aromatase PCR amplifications were performed with the use of the Sybr Green Supermix (Bio-Rad). The primer sequences specific to PI.4 (sense, GTGACC-AACTGGAGCCTG; antisense, CAGGAATCTGCCGTG-GGAGA) and PII (sense, GCAACAGGAGCTATAGAT; antisense, CAGGAATCTGCCGTGGGAGA) were as previously described (McInnes *et al.* 2008). The data were normalized to β-actin levels.

Statistical analysis

Mean activities were calculated from at least three independent experiments done in triplicate. The Student's two-tailed *t*-test was used to determine the significant differences between two groups. $P<0{\cdot}05$ is considered statistically significant.

Results

MSA inhibits aromatase activation

As described in the Introduction, promoters PI.4 and PII can be activated by glucocorticoid and cAMP–protein kinase A signaling respectively. We first assessed the effect of MSA on aromatase activity induced by glucocorticoid and cAMP–protein kinase A. SGBS and KGN cells were chosen as the cell models for our study because their aromatase expression is driven mainly by promoter PI.4 or PII respectively (Ghosh *et al.* 2008, McInnes *et al.* 2008). Consistent with previous reports (McInnes *et al.* 2008, Ohno *et al.* 2009), the basal activity of aromatase was almost undetectable in both cell models, and the activity was induced respectively by Dex, a synthetic glucocorticoid, or FSK, a highly specific activator of adenylate cyclase (Fig. 1). Treatment of SGBS cells with MSA led to a dose-dependent inhibition of Dex-induced aromatase activation (Fig. 1A). The inhibitory effect of MSA on FSK induction of aromatase appears to be even more robust (Fig. 1B). A more than 60% inhibition was already evident with 0·6 μM MSA (Fig. 1B).

MSA inhibition of aromatase activation is not mediated at the enzymatic level

Aromatase inhibitors suppress aromatase activity through disrupting the binding of the substrates to aromatase (Chen *et al.* 2007). In order to determine whether the same mechanism underlies MSA inhibition of aromatase activation, we assessed the direct effect of MSA on the enzymatic activity of aromatase. The experiment was done by adding MSA to the culture at the same time as ^{3}H-androstenedione so that MSA was not present when aromatase expression was induced

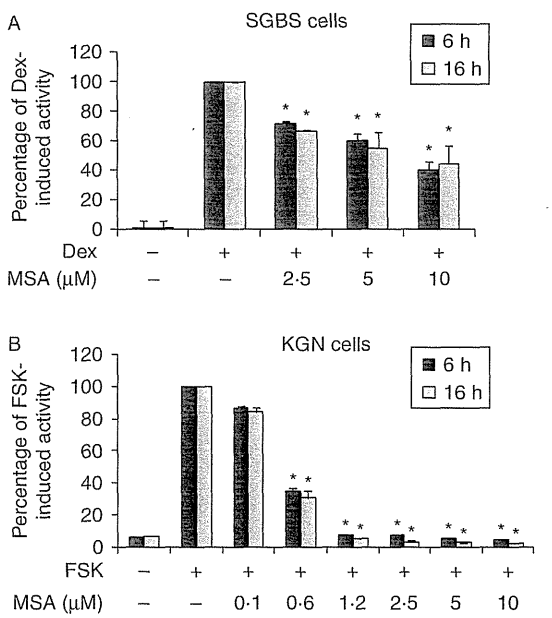

Figure 1 Aromatase activity in (A) SGBS and (B) KGN cells in response to MSA treatment. Cells were treated with or without inducers in the presence or absence of MSA in phenol red-free medium for 6 or 16 h. The inducers and MSA were then removed, and cells were incubated with ^{3}H-androstenedione for an additional 4 h. $^{*}P<0{\cdot}05$ compared to inducer-treated sample.

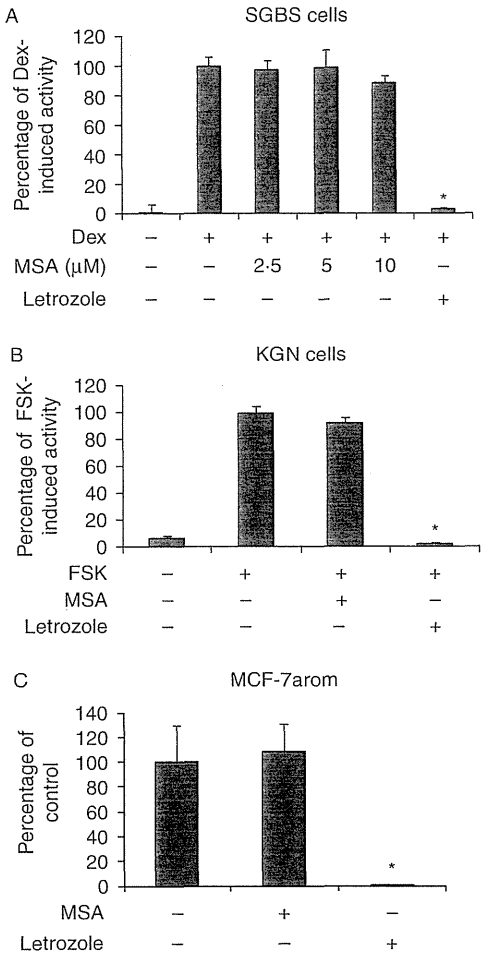

Figure 2 Direct effect of MSA on aromatase enzymatic activity in (A) SGBS, (B) KGN, and (C) aromatase-transfected MCF-7arom cells. Cells were treated with inducers in phenol red-free medium for 16 h. Inducers were then removed, and cells were incubated with ^{3}H-androstenedione in the presence or absence of MSA or letrozole for an additional 4 h. *$P<0·05$ compared to inducer-treated sample.

by Dex or FSK. We used the aromatase inhibitor letrozole as the positive control. The data, as presented in Fig. 2A and B, showed that, in both SGBS and KGN cells, while letrozole almost completely abolished the activity of aromatase, no significant change of aromatase activity was detected after MSA treatment. We also determined the response of an aromatase-overexpressing stable transfectant, MCF-7arom, to MSA treatment. The expression of aromatase in MCF-7arom cells is driven by a constitutive promoter. Consistently, we did not observe modulation of aromatase activity by MSA in these cells (Fig. 2C). The data were apparently different from that obtained when MSA was added to the culture together with the aromatase expression inducers (Fig. 1), indicating that the effect of MSA on aromatase is not mediated through affecting aromatase enzymatic activity.

MSA downregulates PI.4- and PII-mediated aromatase expression

To unravel the mechanism by which MSA inhibits aromatase activation, we assessed MSA modulation of aromatase protein. As shown in Fig. 3A, 0·6 μM MSA inhibited FSK-induced aromatase protein by more than 50%, and 2·5 μM MSA totally blocked the induction. We also examined the effect of MSA on Dex-induced aromatase protein expression in SGBS cells. However, the low level of aromatase expression in SGBS cells, even after Dex induction, was under the detection limit of aromatase western blot analysis. We next characterized the effect of MSA on the level of total aromatase mRNA by real-time RT-PCR using primers recognizing all aromatase transcripts. As shown in Fig. 3B and C, MSA significantly suppressed Dex and FSK induction of aromatase mRNA, and the effect on FSK-induced expression was even more pronounced. In order to confirm that the decrease in total aromatase mRNA was due to suppression of transcription from promoter PI.4 or PII, we repeated the real-time RT-PCR analysis using primers specific to PI.4 or PII. The results, as shown in Fig. 4, are in great concordance with that presented in Fig. 3B and C. Taken together, the data indicated that MSA suppressed aromatase activation through downregulating promoter PI.4- and PII-driven aromatase mRNA expression.

Discussion

Elevated circulating estrogen levels, as a result of increased peripheral aromatization of androgens, have been indicated to underlie the association between obesity and a higher risk of breast cancer in postmenopausal women (Brown *et al.* 2009,

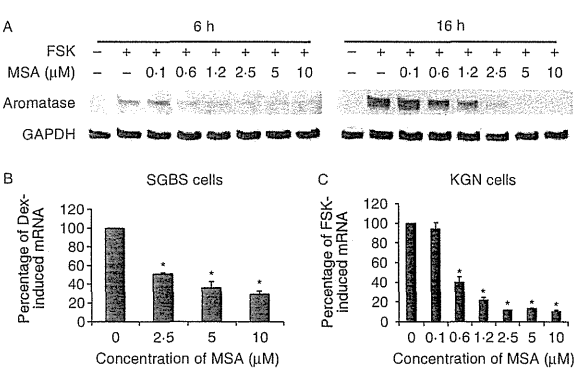

Figure 3 Effect of MSA on aromatase protein expression in KGN cells (A) and aromatase mRNA expression in SGBS (B) and KGN cells (C). Cells were treated with inducers in the presence or absence of MSA in phenol red-free medium containing charcoal-stripped FBS for 6 or 16 h for protein analysis and for 3 h for aromatase mRNA expression. *$P<0·05$ compared to inducer-treated sample.

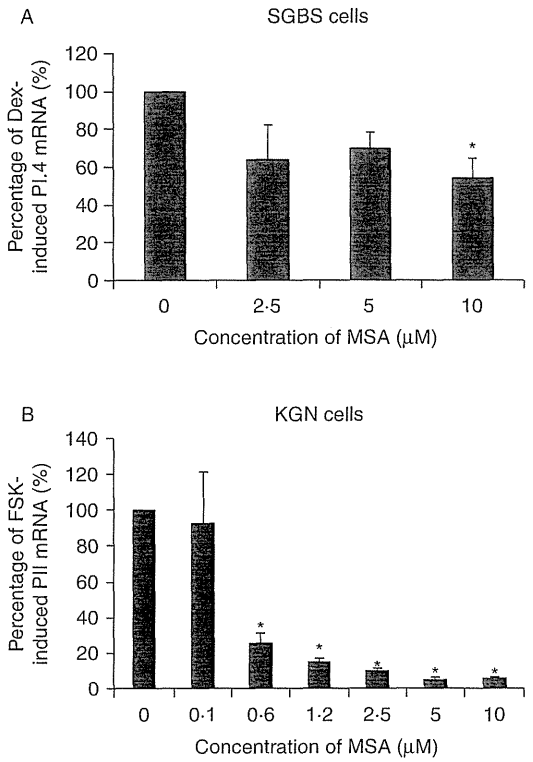

Figure 4 MSA effect on promoter (A) PI.4- and (B) PII-specific aromatase expression in SGBS and KGN cells respectively. Cells were treated with inducers in the presence or absence of MSA in phenol red-free medium containing charcoal-stripped FBS for 3 h. *$P<0{\cdot}05$ compared to inducer-treated sample.

Brown & Simpson 2010). In the present study, we showed that both PI.4- and PII-driven aromatase transcription can be efficiently suppressed by MSA, leading to a marked down-regulation of aromatase mRNA, protein, and thereby activity. Considering the low-cost and low-toxicity nature of MSA, the data provide a strong rationale for the further development of MSA as a breast cancer chemopreventive agent for obese postmenopausal women.

Our data on MSA suppression of PII- and PI.4-driven aromatase expression also suggest a role of MSA in reducing breast intratumoral estrogen level. In postmenopausal breast cancer patients, tumor concentration of estrogens has been reported to be ~10 times the concentration in plasma (van Landeghem *et al.* 1985, Bulun *et al.* 1993, Agarwal *et al.* 1996, Miller *et al.* 1997). This is attributable to the heterotypic interaction between breast tumor cells and tumor adjacent adipose stromal cells (Bulun *et al.* 2005). In response to estrogen stimulation, breast tumor cells secrete large amounts of cytokines, such as tumor necrosis factor α and interleukin 11, to inhibit adipogenic differentiation of adjacent stromal cells (Crichton *et al.* 1996, Meng *et al.* 2001). This leads to an upregulated PII promoter activity (Irahara *et al.* 2006) and thereby an elevated local aromatase expression and estrogen level (Meng *et al.* 2001), thus creating a localized, growth-stimulatory environment for tumor cells. In addition, aromatase expression has also been detected in breast tumor cells, although at a much lower level compared to the adipose stromal cells (Miki *et al.* 2007). Promoter PII is also a main promoter driving the expression of aromatase in breast tumor cells (Agarwal *et al.* 1996). Therefore, reducing intratumoral production of estrogen may represent an additional novel mechanism of MSA anticancer action.

In fact, we have sought to study the effect of MSA on aromatase expression in breast cancer cell lines. However, none of the breast cancer cell lines that we have tested, including MCF-7, T47D, and MDA-MB-468, have detectable aromatase expression even after inducer treatment. Whether cultured breast cancer cell lines express a detectable amount of aromatase is still debatable. While some groups were able to detect aromatase expression in cell lines such as MCF-7, T47D, and MDA-MB-468 (Kijima *et al.* 2006, Miki *et al.* 2007, Ciolino *et al.* 2011), others could not (Sanderson *et al.* 2001, Heneweer *et al.* 2005). Nevertheless, the data that we obtained from KGN cells should be applicable to breast tumor cells as PII-mediated expression is regulated mainly by the cAMP–protein kinase A pathway in both cell types (Zhao *et al.* 1996*a*, Ghosh *et al.* 2008).

A number of transcription factors have been implicated in PII regulation, including LRH-1, CREB, CRTC2, ATF2, SF-1, C/EBPs, Jun, and several orphan nuclear receptors (Zhou *et al.* 2001, Clyne *et al.* 2002, Yang *et al.* 2002, Sofi *et al.* 2003, Ghosh *et al.* 2008, Kijima *et al.* 2008, Brown *et al.* 2009). PI.4 is a TATA-less promoter that contains a glucocorticoid response element, Sp1-binding site, and an interferon-γ activation site element (Zhao *et al.* 1996*b*). The JAK/STAT signaling pathway has been reported to be involved in PI.4 regulation (Zhao *et al.* 1995). MSA has been shown to alter the expression levels of a number of proteins in stromal cells, including cAMP-responsive element-binding protein 6 (CREB6) (Jiang *et al.* 1999, Tsavachidou *et al.* 2009, Zhang *et al.* 2010). We are currently investigating the effect of MSA on signal transduction from the cAMP–protein kinase A and glucocorticoid receptor/JAK/STAT pathways to elucidate the mechanisms by which MSA inhibits aromatase expression.

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

Y D, R G, L Z, B G R, and Q Y designed the research; R G and X L conducted the study; R G, B G R, Q Y, and Y D wrote the paper; M W supplied SGBS cells; D P E supplied the aromatase antibody; Y N and T Y supplied KGN cells; Y D and Y Q had primary responsibility for the final content.

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Gradual Loss of ACTH Due to a Novel Mutation in *LHX4*: Comprehensive Mutation Screening in Japanese Patients with Congenital Hypopituitarism

Masaki Takagi[1,2], Tomohiro Ishii[1], Mikako Inokuchi[1], Naoko Amano[1], Satoshi Narumi[1], Yumi Asakura[3], Koji Muroya[3], Yukihiro Hasegawa[2], Masanori Adachi[3], Tomonobu Hasegawa[1]*

1 Department of Pediatrics, Keio University School of Medicine Tokyo, Japan, **2** Department of Endocrinology and Metabolism, Tokyo Metropolitan Children's Medical Center, Tokyo, Japan, **3** Department of Endocrinology and Metabolism, Kanagawa Children's Medical Center, Kanagawa, Japan

Abstract

Mutations in transcription factors genes, which are well regulated spatially and temporally in the pituitary gland, result in congenital hypopituitarism (CH) in humans. The prevalence of CH attributable to transcription factor mutations appears to be rare and varies among populations. This study aimed to define the prevalence of CH in terms of nine CH-associated genes among Japanese patients. We enrolled 91 Japanese CH patients for DNA sequencing of *POU1F1, PROP1, HESX1, LHX3, LHX4, SOX2, SOX3, OTX2,* and *GLI2*. Additionally, gene copy numbers for *POU1F1, PROP1, HESX1, LHX3,* and *LHX4* were examined by multiplex ligation-dependent probe amplification. The gene regulatory properties of mutant LHX4 proteins were characterized *in vitro*. We identified two novel heterozygous *LHX4* mutations, namely c.249-1G>A, p.V75I, and one common *POU1F1* mutation, p.R271W. The patient harboring the c.249-1G>A mutation exhibited isolated growth hormone deficiency at diagnosis and a gradual loss of ACTH, whereas the patient with the p.V75I mutation exhibited multiple pituitary hormone deficiency. *In vitro* experiments showed that both *LHX4* mutations were associated with an impairment of the transactivation capacities of *POU1F1* and*αGSU*, without any dominant-negative effects. The total mutation prevalence in Japanese CH patients was 3.3%. This study is the first to describe, a gradual loss of ACTH in a patient carrying an *LHX4* mutation. Careful monitoring of hypothalamic–pituitary -adrenal function is recommended for CH patients with *LHX4* mutations.

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* E-mail: thaseg@a6.keio.jp

Introduction

The proliferation and terminal differentiation of the anterior pituitary gland is strongly influenced by the precise spatial and temporal expression of transcription factors [1–3]. Mutations in these transcription factors often result in various types of congenital hypopituitarism (CH) [1–3]. Although previous studies have shown that these transcriptional factor mutations are rare among CH patients and that the mutation prevalence varies among populations, only a few genetic screening studies have been conducted. Graaff *et al.* identified a single patient with a *POU1F1* mutation from a study population of 79 multiple pituitary hormone deficiency (MPHD) patients (1.2%) in The Netherlands [4], and Dateki *et al.* reported one patient harboring an *LHX4* gross deletion from a cohort of 71 MPHD patients (1.4%) in Japan [5]. On the other hand, Reynaud *et al.* reported a mutation prevalence of 13.3% in a study population of 165 MPHD patients from the international GENHYPOPIT network [6]. Approximately 90% of the mutations identified in this report were *PROP1* common mutations (149delGA and 296delGA). Although the 296delGA mutation represents a mutational hot spot within the *PROP1* gene rather than a common founder mutation [7], studies from other ethnic groups often report a low prevalence of *PROP1* mutations [8,9].

This study aimed to determine the prevalence of transcription factor mutations in Japanese CH patients with PCR-based sequencing of nine CH-associated genes, namely *POU1F1, PROP1, HESX1, LHX3, LHX4, SOX2, SOX3, OTX2,* and *GLI2*. Additionally, we examined the gene copy numbers of *POU1F1, PROP1, HESX1, LHX3,* and *LHX4* by multiplex ligation-dependent probe amplification (MLPA).

Materials and Methods

Subjects

This study population consisted of 91 patients with GH-treated CH. The inclusion criteria were as follows: 1) short statue with severe GH deficiency (GH peak < 3 ng/mL) confirmed by hypoglycemic provocation test, and 2) anterior pituitary hypoplasia as detected by brain magnetic resonance imaging (MRI). We excluded any CH patients of known cause, such as a brain tumor or brain surgery from this study. Patients or parents of patients under 18 years of age gave their written informed consent to

Table 1. Endocrine phenotype of 91 probands screened for 9 genes.

	No. (%) with deficiencies of			
	GH	TSH	ACTH	LH/FSH
IGHD (n = 14)	14(100)			
MPHD (n = 77)	77(100)	61(79)	34(44)	19(24)

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participate in this study, which was approved by the Institutional Review Board of Keio University School of Medicine and the Institutional Review Board of Kanagawa Children's Medical Center.

Endocrinological investigations

Hormonal assays were performed using several commercial RIA kits, and normal values for each center were taken into account. The results of biochemical investigations at diagnosis were recorded including basal free thyroxine (fT4), TSH, cortisol and ACTH levels, their peaks in response to pituitary stimulation tests. The patients were evaluated for serum GH level after two consecutive classical provocative tests (with arginine or insulin). GH peaks <6 ng/mL after stimuli support a diagnosis of GHD. GH peak < 3 ng/mL by hypoglycemic provocation test define severe GHD. A diagnosis of TSH deficiency was made if serum fT4 concentration was under the normal level (fT4 < 1.0 ng/dL) with inadequate low serum TSH concentration. Cortisol peaks <17 μg/dL by hypoglycemic provocation tests define ACTH deficiency. FSH–LH deficiency was diagnosed on the basis of delayed or absent pubertal development and inadequate increase in serum FSH and LH in response to LHRH.

Imaging investigations

MRI included T1 and T2 weighted high-resolution pituitary imaging through the hypothalamo-pituitary axis (T1 sagittal 3-mm slices, T1 and T2 coronal 3-mm slices). Details noted included the size of the anterior pituitary, position of the posterior pituitary signal, presence and morphology of the optic nerves, optic chiasm, pituitary stalk, septum pellucidum, and corpus callosum.

Mutation screening

For all patients, regardless the phenotype/pituitary MRI findings, we analyzed all coding exons and flanking introns of *POU1F1, PROP1, HESX1, LHX3, LHX4, OTX2, SOX2, SOX3*, and *GLI2* by PCR-based sequencing. We screened for deletion/duplication involving *POU1F1, PROP1, HESX1, LHX3*, and *LHX4* by MLPA analyses (SALSA MLPA KIT P216; MRC-Holland, Amsterdam, The Netherlands). We tested any detected sequence variations against 150 Japanese control subjects.

RT-PCR

For mRNA analysis of the *LHX4* c.249-1G>A mutation, total RNA was extracted from Epstein-Barr virus-transformed lymphocytes derived from the propositus of pedigree 1. The cDNA produced from reverse transcription of RNA was subjected to PCR amplification using primers encompassing exons 2 to 4, and were subsequently processed for direct sequencing.

Functional studies

We performed functional studies on the two novel *LHX4* mutations (p.R84X and p.V75I). To generate LHX4 expression vectors, LHX4 cDNA was cloned into pCMV-myc and pEGFP-N1 (Clontech, Palo Alto, CA). We introduced the two mutations by site-directed mutagenesis, using the PrimeSTAR Mutagenesis Basal Kit (TaKaRa, Otsu, Japan). The luciferase reporter vectors were constructed by inserting the promoter sequences of *POU1F1 (PIT1)*,*αGSU* into a pGL3 basic vector (Promega, Madison, WI). A transactivation assay was performed using dual-luciferase reporter assay system (Promega) on COS7 and GH3 cells. For western blot analyses, we harvested COS7 cells transfected with the myc-tagged LHX4. Western blotting was performed with a mouse anti-myc monoclonal antibody (Invitrogen, Carlsbad, CA). For subcellular localization analyses, we visualized and photographed COS7 cells transfected with GFP-tagged LHX4 using a Leica TCS-SP5 laser scanning confocal microscope (Leica, Exton, PA). The sequences of the biotin-labeled doublestranded oligonucleotide used as probe in the EMSA experiment was 5′-GTATGAATCATTAATTGA-CAACATATTTTC-3′, as described previously [10]. The probes were detected with the Lightshift chemiluminescent EMSA kit (Pierce) according to the manufacturer's instruction.

Results

Patient details

Of the 91 patients, on the basis of hormonal deficiencies, 14 were determined to have isolated GH deficiency (IGHD), whereas 77 were MPHD. Detailed endocrine phenotype was available in all of the 91patients (Table 1). Results of the MRI scans were available in all patients with IGHD and MPHD. Details regarding the structural abnormalities of the hypothalamo-pituitary axis on neuroimaging in the probands are shown in Table 2. Among 77 MPHD patients, 12 were diagnosed as Septo-optic dysplasia.

Mutation screening

We identified two novel heterozygous *LHX4* mutations, namely c.249-1G>A, expected to cause exon skipping, and c.223G>A (p.V75I), and one common heterozygous *POU1F1* mutation,

Table 2. Results of MR scans of probands screened for 9 genes.

	Morphology of						
	Anterior pituitary	Posterior pituitary			Stalk		
	Hypoplasia	Normal	Ectopic	Absent	Normal	Invisible	Thin
IGHD (n = 14)	14	5	9	0	4	5	5
MPHD (n = 77)	77	24	51	2	23	25	29
Total (n = 91)	91	29	60	2	27	30	34

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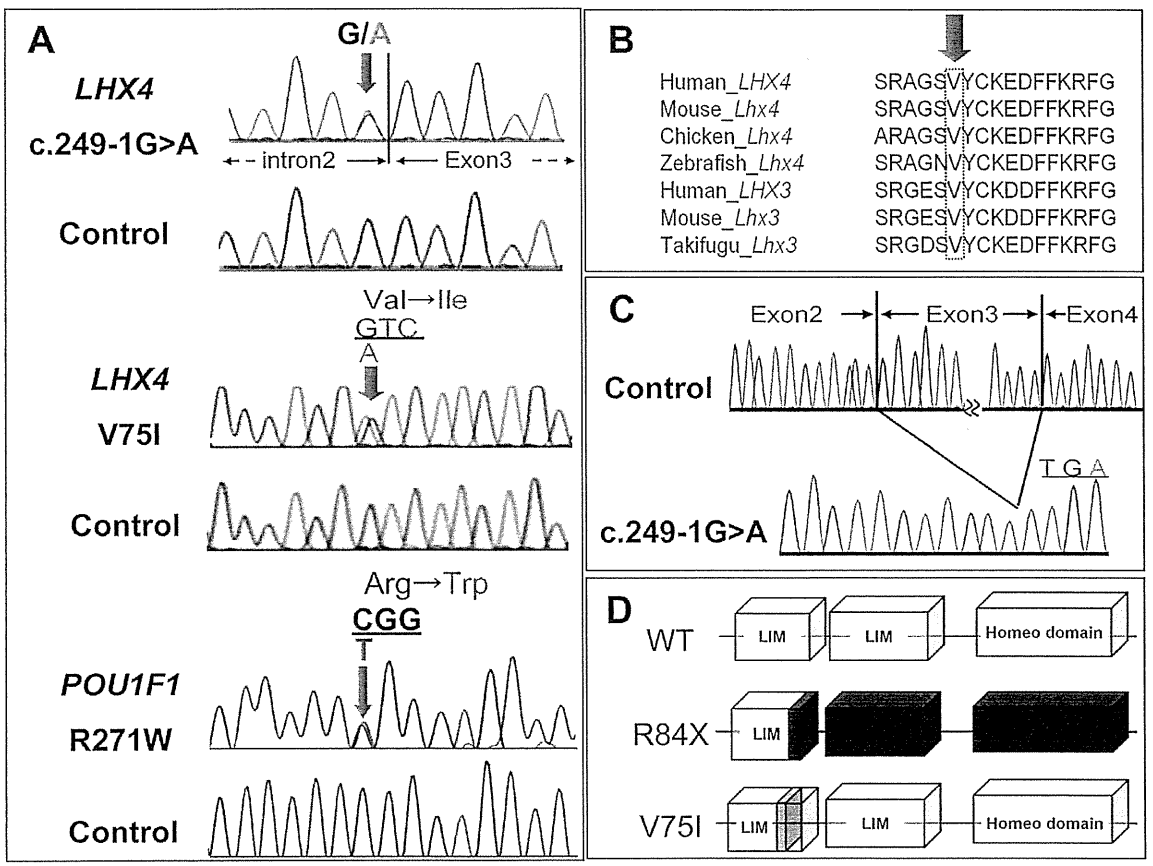

Figure 1. Identification of sequence variations of *LHX4* and *POU1F1*. *A*, Partial sequences of PCR products of the patients are shown. The upper chromatogram represents a heterozygous G to A substitution in the splice acceptor site of exon3. The middle chromatogram represents a heterozygous substitution of isoleucine (ATC) in place of valine (GTC) at codon 75. The arrow indicates the mutated nucleotide. The lower chromatogram represents a heterozygous substitution of tryptophan (TGG) in place of arginine (CGG) at codon 271. The arrow indicates the mutated nucleotide. *B*, Homology study showed valine at codon 75 is highly conserved through species in LHX4 and LHX3. C, Identification of exon3 skipping in the LHX4 cDNA derived from propositus of pedigree 1. LHX4 transcript with a deleted exon 3 creates a premature stop codon at the beginning of the remaining exon 4 (p.R84X). *D*, Schematic diagrams of the LHX4 protein. LHX4 cDNA encodes two LIM domains and one homeodomain. LHX4 with a p.R84X mutation results in the deletion of one of the two LIM domains and the entire homeodomain. Val75 is located within the first LIM domain.
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c.811C>T (p.R271W) [11] (FIG. 1A). The V75 in LHX4 is evolutionarily highly conserved (FIG. 1B), and these two *LHX4* mutations were not detected in any of the 150 healthy Japanese controls. We detected no gross or exon-level deletions/duplications using the MLPA analyses. For 14 IGHD patients, we additionally analyzed all coding exons and flanking introns of *GH1*, and *GHRHR* by PCR-based sequencing and MLPA (SALSA MLPA KIT P216 included all exons of *GH1* and *GHRHR*), failing to detect any sequence variation.

RT-PCR

The RT-PCR generated a product of smaller size than that obtained from a control sample. Sequencing revealed that it corresponded to a LHX4 transcript skipping exon 3 (FIG. 1C). If translated, this abnormal transcript would generate a protein lacking one of the two LIM domains (LD) and the entire homeodomain (HD), p.R84X (FIG. 1D).

Clinical phenotypes of the mutation carriers

Pedigree 1: *LHX4* c.249-1G>A (FIG. 2A). The propositus was a 16-year-old Japanese female, who was born at 39 weeks of gestation after an uncomplicated pregnancy and delivery. At birth, her length was 51.0 cm (1.2 SD) and weight 3.3 kg (0.6 SD). She was referred to us at 5 years of age because of short stature. Her height was 92.4 cm (-3.6 SD). Endocrine studies indicated that the patient had IGHD (Table 3). Brain MRI showed anterior pituitary hypoplasia, with a visible but thin stalk, and an ectopic posterior pituitary gland (EPP). No other central nervous system abnormalities were visualized. Recombinant human GH therapy was started at age 6. Her growth was responded well to GH replacement. Although she had no definite episode of adrenal insufficiency, longitudinal data showed that her blood cortisol peak, after stimulation by hypoglycemia with insulin tolerance tests, decreased gradually with age (20.5, 17.5, 16.4, and 10.0 μg/dL, at ages of 5, 13, 14, and 15 years, respectively, Ref. >17 μg/

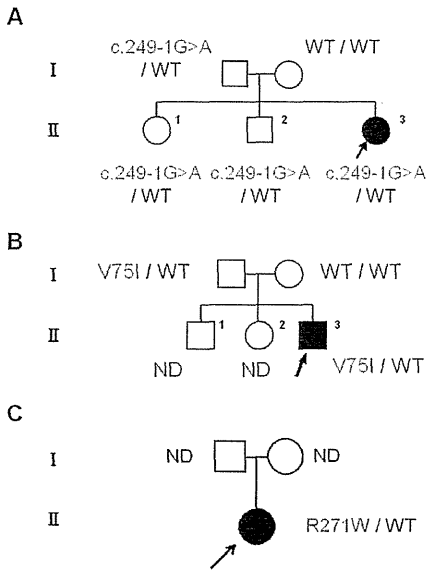

Figure 2. The pedigrees of the affected families. *A–C*, Pedigrees of families 1–3. Arrow indicates the propositus. ND: not determined.
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dL [12]), indicating of a gradual loss of ACTH. Follow-up MRI showed no changes as compared with the initial finding.

The father of the patient was 153.0 cm (-2.9 SD) tall, and the mother was 160.8 cm (0.5SD) tall. The elder brother and sister of the patient, both reached normal adult heights of 171.7 cm (0.2 SD) and 152.1 cm (-1.3 SD), respectively. Genetic analyses showed that the propositus, siblings and father carried the heterozygous *LHX4* c.249-1G>A mutation. No family members had any baseline hormonal abnormalities (Table 4).

Pedigree 2: *LHX4* p.V75I (FIG. 2B). The propositus was a 13-year-old Japanese male born at 41 weeks of gestation after an uncomplicated pregnancy and delivery. At birth, his length was 51.0 cm (1.0 SD) and weight 3.3 kg (0.7 SD). He was referred to us at 3 months of age because of a micropenis and bilateral cryptorchidism. He had undetectable plasma testosterone and LH levels, indicating hypogonadotropic hypogonadism. Severe growth failure was observed at the age of 11 months. Hormonal data revealed GH and TSH deficiencies in addition to tentative gonadotropin deficiency (Table 5). Brain MRI exhibited anterior pituitary hypoplasia, poorly developed sella turcica, visible but thin stalk, and EPP. No other central nervous system abnormalities were visualized. Replacement therapy with thyroxine and recombinant human GH was started at the age of 1 year. The patient responded well to GH replacement. At the age of 13 years, he showed small intrascrotal testes (1 ml), no pubic hair (P1), and a microphallus with low concentration of basal testosterone (0.05 ng/mL Ref: 2.0–7.5).

Table 3. Endocrinological findings in Propositus of pedigree 1.

		5yr			15yr			Reference	
	Stimulus	**Basal**		**Peak**	**Basal**		**Peak**	**Basal**	**Peak**
GH (ng/ml)	Insulin	2.7	→	2.9	1.8	→	2.6		>6
TSH (mIU/ml)	TRH	2.88	→	10.01	0.78	→	7.42		10–35
LH (mIU/ml)	LHRH	<0.2	→	2.8	6.7	→	21.2	<0.1[a]	1.93–4.73 [a]
								<0.10–2.65[b]	6.69–22.51 [b]
FSH (mIU/ml)	LHRH	0.5	→	15.5	7.0	→	9.6	0.64–3.03[a]	13.15–46.95[a]
								1.81–7.31[b]	8.58–17.62[b]
PRL (ng/ml)	TRH	10.4	→	19.7	5.7	→	28.1	1.7–15.4	increase 2 times
ACTH (pg/ml)	Insulin	44	→	46	7.3	→	14.9	9.8–27.3	28–130.5
Cortisol (μg/dl)	Insulin	19.1	→	20.5	7.5	→	10.0		>19.8[c]
									>17.0[d]
IGF-1 (ng/ml)		70.1			241			74–230[e]	
								262–510[f]	
Free T4 (ng/dl)		1.1			1.0			1.0–1.95	
Free T3 (pg/ml)		4.2			2.1			2.23–5.30	
Estradiol (pg/ml)					28			12.3–170[g]	

The conversion factors to the SI unit are as follows: GH 1.0 (μg/liter), LH 1.0 (IU/liter), FSH 1.0 (IU/liter), TSH 1.0 (mIU/liter), prolactin 1.0 (μg/liter), ACTH 0.22 (pmol/liter), cortisol 27.59 (nmol/liter), IGF-I 0.131 (nmol/liter), free T4 12.87 (pmol/liter), free T3, 1.54 (pmol/liter), and estradiol 3.671 (pmol/liter).
[a]Reference data of pre-pubertal Japanese girls [22]
[b]Reference data of pubertal (Tanner 2–3) Japanese girls [22]
[c]Reference data of UK children (younger than 10 years) [23]
[d]Reference data of UK children (older than 10 years) [23]
[e]Reference data of Japanese girls (5–7 years old) [24]
[f]Reference data of Japanese girls (15–17 years old) [24]
[g]Reference data of Japanese girls (15 years old) [25]
doi:10.1371/journal.pone.0046008.t003

Table 4. Endocrinological findings (baseline) in Family members of pedigree 1.

	Father	Mother	Brother	Sister	Reference (Adult)
GH (ng/ml)	0.7	3.2	0.5	0.4	0–23
IGF-1 (ng/ml)	110.0	156.0	357.0	276.0	Male: 41–369
					Female: 73–542
TSH (μU/ml)	0.77	1.60	0.50	0.94	0.3–3.50
Free T4 (ng/dl)	1.1	1.1	1.4	1.3	1.09–2.55
Free T3 (pg/ml)	2.5	2.6	3.1	3.1	3.23–5.11
LH (mIU/ml)	4.8	7.4	2.1	6.9	Male: 2.2–8.4
					Female: 1.4–15[a]
FSH (mIU/ml)	2.9	4.3	2.3	7.9	Male: 1.8–12
					Female: 3–10[a]
PRL (ng/ml)	11.2	11.2	7.8	5.5	Male: 1.5–9.7
					Female: 1.4–14.6
ACTH (pg/ml)	14	12	15	20	7.2–63.3
Cortisol (μg/dl)	8.2	6.3	10.3	10.3	7.6–21.4
Estradiol (pg/ml)		397		23	Female: 11–230[a]
Testosterone (ng/ml)	5.19		5.56		Male: 2.01–7.50

[a]Follicular phase

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The patient's father was 160.5 cm (-1.8 SD) tall. Genetic analyses showed that the propositus and father carried the same heterozygous *LHX4* p.V75I mutation. No other family member was available for genetic studies. Evaluation of the hormonal data for the father was refused.

Pedigree 3: *POU1F1* p.R271W (FIG. 2C). The propositus was a 28-year-old Japanese female, who was born at 37 weeks of gestation after an uncomplicated pregnancy and delivery. At birth, her length was 48.0 cm (-0.2 SD) and weight 2.6 kg (-1.0 SD). She was referred to us at 2 years of age because of severe short stature (-4.5 SD). Endocrine studies indicated that the patient had complete GH and PRL deficiencies and partial TSH deficiency (free T4 0.8 ng/dl, Ref. >1.0, with inadequate low TSH). Brain MRI at the age of 7 years exhibited anterior pituitary hypoplasia, normal stalk, and normal posterior pituitary gland. No other

Table 5. Endocrinological findings in Propositus of pedigree 2.

		11month			8yr			Reference	
	Stimulus	Basal		Peak	Basal		Peak	Basal	Peak
GH (ng/ml)	Insulin	1.1	→	0.9	0.6	→	0.6		>6
TSH (mIU/ml)	TRH	0.56	→	6.81	2.00	→	10.81		10-35
LH (mIU/ml)	LHRH	0.3	→	0.8	0.2	→	2.3	<0.1[a]	<0.10-4.29 [a]
FSH (mIU/ml)	LHRH	2.1	→	2.6	1.5	→	7.4	0.46-1.43[a]	5.38-11.67[a]
Testosterone (ng/ml)	HCG				<0.05		0.17		>1.2[a]
PRL (ng/ml)	TRH	5.6	→	10.1	7.7	→	13.0	1.7-15.4	increase 2 times
ACTH (pg/ml)	Insulin	44	→	170	44	→	50	9.8-27.3	28-130.5
Cortisol (μg/dl)	Insulin	31.0	→	38.4	13.4	→	17.2	5-20	>19.8[b]
IGF-1 (ng/ml)		6.9			157			18-150 [c]	
								50-356[d]	
Free T4 (ng/dl)		1.1			1.1			1.01-1.95	
Free T3 (pg/ml)		4.4			3.9			2.23-5.30	

The conversion factors to the SI unit are as follows: GH 1.0 (μg/liter), TSH 1.0 (mIU/liter), LH 1.0 (IU/liter), FSH 1.0 (IU/liter), testosterone, 0.035 (nmol/liter), prolactin 1.0 (μg/liter), ACTH 0.22 (pmol/liter), cortisol 27.59 (nmol/liter), IGF-I 0.131 (nmol/liter), free T4 12.87 (pmol/liter), and free T3, 1.54 (pmol/liter).

[a]Reference data of pre-pubertal Japanese boys (younger than 10 years) [22]

[b]Reference data of UK children (younger than 10 years) [23]

[c]Reference data of Japanese boys (younger than 1 years old) [24]

[d]Reference data of Japanese boys (7-9 years old) [24]

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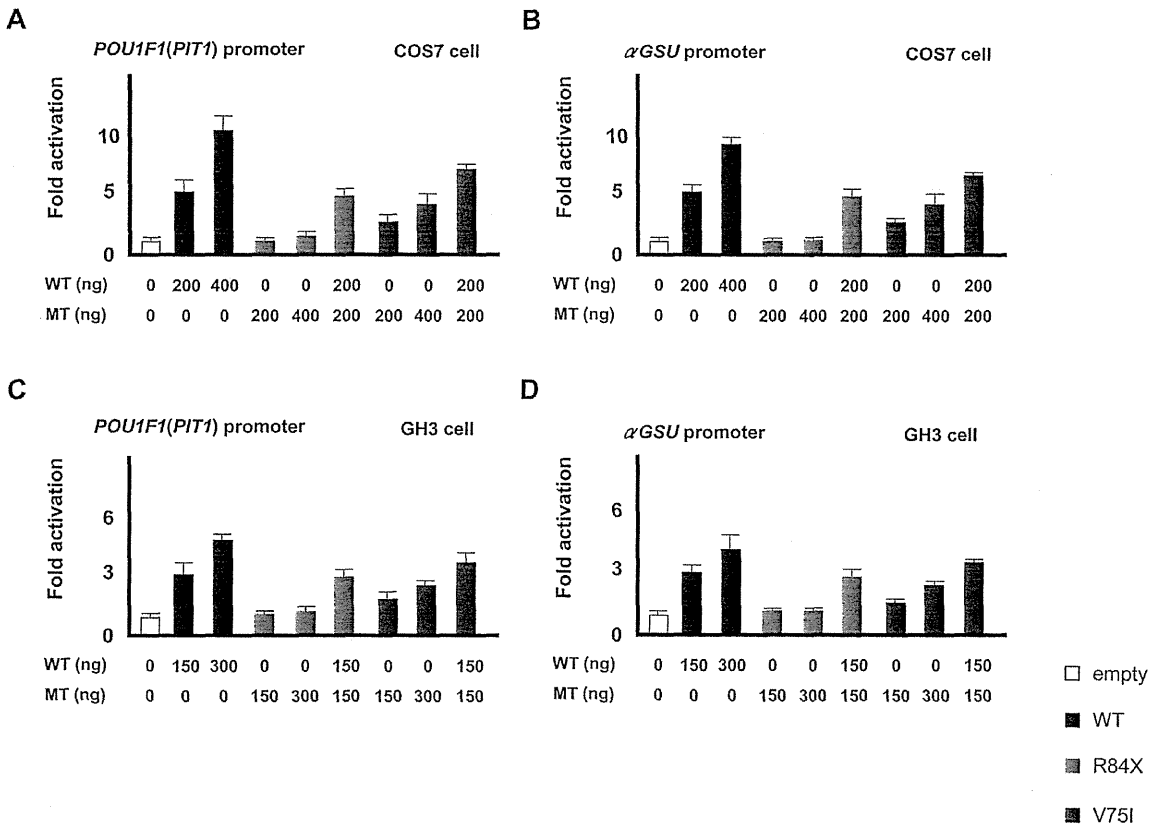

Figure 3. Transactivation assays of R84X and V75I LHX4 using *POU1F1*(*PIT1*) and*αGSU* reporter. *A and B*: COS7 cells were cotransfected with the pRL-CMV internal control vector, indicated amount (nanograms) of the effector plasmids, and the *POU1F1*(A) or*αGSU* (B) reporter. The data are the mean ± s.e.m. of at least three independent experiments performed in triplicate transfections. The white, black, red, and blue bars indicate the data of the empty expression vectors, expression vectors with wild type (WT) LHX4, expression vectors with R84X LHX4, and V75I LHX4, respectively. R84X LHX4 exhibited markedly reduced transactivation, whereas V75I LHX4 retained partial activity. The two mutants did not exhibit any dominant negative effect. The data are mean ± SEM of at least three independent experiments performed in triplicate transfections. *C and D*: GH3 cells were cotransfected with the pRL-CMV internal control vector, indicated amount (nanograms) of the effector plasmids, and the *POU1F1*(C) or*αGSU* (D) reporter.
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central nervous system abnormalities were visualized. The patient responded well to GH replacement.

Functional studies

Both in COS7 and GH3 cells, wild type LHX4 stimulated transcription of the *POU1F1* and*αGSU* reporters in a dose-dependent manner. R84X LHX4 had markedly reduced transactivation, whereas V75I LHX4 retained partial activity (FIG. 3A-D). The two mutants had no dominant negative effect. Western blot analysis showed that the expression of V75I LHX4 was comparable to that of the wild type, whereas R84X LHX4 was not detected (FIG. 4A). The V75I LHX4 mutant localized to the nucleus (FIG. 4B). WT LHX4 showed specific binding to the elements, which were competed by excess amount of (200 times) cold competitors. The V75I LHX4, which has an intact HD, bound with similar or slightly high efficiency to the WT LHX4 (FIG. 4C).

Discussion

In the present study, our mutation prevalence data (three mutation carriers in a total of 91 CH patients: 3.3%) is comparable with earlier report of Graaff *et al.* (1.2%) [4] or Dateki *et al* (1.4%) [5]. This study enrolled CH patients that fulfilled two definite inclusion criteria: 1) severe GH deficiency (GH peak < 3 ng/mL) confirmed by hypoglycemic provocation tests, which included IGHD and MPHD, and 2) anterior pituitary hypoplasia based on brain MRI. The subjects included in the two previous reports were diagnosed with MPHD and the reports of Dateki *et al.* did not describe any specific inclusion criteria. As *PROP1* common mutations (149delGA and 296delGA) are rare in Japan, our prevalence data were lower than that of Reynaud *et al.* [6]. These previous studies did not include screening for *SOX2*, *SOX3*, *OTX2* and *GLI2* (although the study by Dateki *et al.* included *SOX3* and *OTX2*), thus this study serves as the first report to include these genes. Despite extending the range of our genetic screening, our results imply the rarity of pathological abnormalities in the

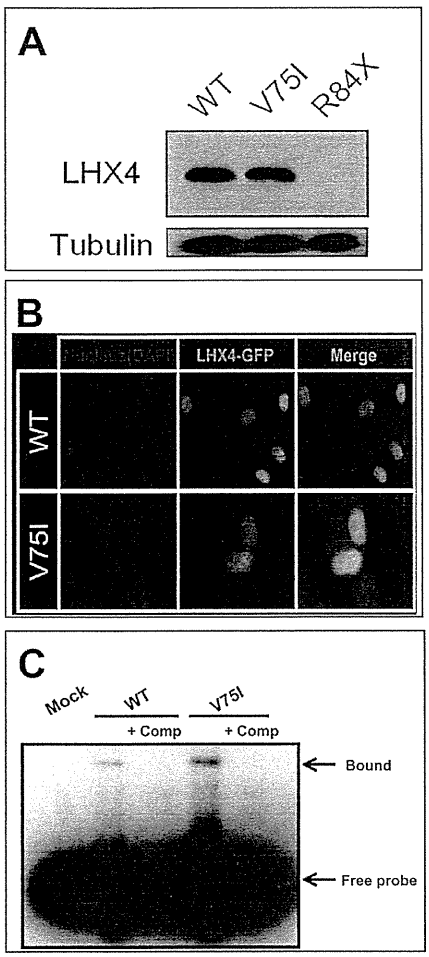

Figure 4. Functional characterization of two mutant LHX4. *A*, Protein expression level of myc-tagged WT and two LHX4 mutants was assessed by western blot using a monoclonal anti-myc antibody. The expression of V75I LHX4 was comparable to that of WT, whereas R84X LHX4 was not detected. Tubulin was used as a control. *B*, Subcellular localization analysis. For subcellular localization analyses, we visualized and photographed COS7 cells transfected with GFP-tagged LHX4 using a Leica TCS-SP5 laser scanning confocal microscope, after mounting the cells in Vectashield-DAPI solution. The WT and V75I LHX4 are localized to the nucleus. *C*, EMSA experiments. WT LHX4 showed specific binding to the elements, which was competed by excess amount of (200 times) cold competitors. The V75I LHX4, which has an intact HD, bound with similar or slightly high efficiency to the WT LHX4.
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currently known genes responsible for CH. Further studies are required to understand the pathogenesis of CH.

To date, eight families carrying a *LHX4* mutation have been reported [5,13–17]. We identified two novel mutations in *LHX4* (c.249-1G>A, p.V75I). Although both mutations were associated with impaired transactivation of *POU1F1* and*αGSU* without dominant-negative effects, indicating haploinsufficiency, the mechanism behind the loss of function resulting from these two mutations seems to be different. We did not detect R84X LHX4 on western blotting, indicating that the protein expression is markedly reduced due to the protein's instability. On the other hand, western blotting, visualization of subcellular localization, and DNA binding test revealed no significant difference between the wild type and V75I LHX4 variant. Val75 is a highly conserved amino acid located in the LD (FIG. 1C), which is important for protein-protein interaction, suggesting that substitution of Val75 to Ile results in defective interactions with transcriptional cofactors.

A striking finding of our report is that the propositus, who carried the c.249-1G>A *LHX4* mutation, exhibited a gradual loss of ACTH. Although late onset ACTH deficiency is well known in CH patients with *PROP1* mutations [18–20] and LHX3 [21], our study showed, for the first time, that a gradual loss of ACTH should be a point of concern among CH patients with *LHX4* mutations. Thus, this study suggests careful follow-up monitoring of the hypothalamic-pituitary-adrenal function in CH patients with *LHX4* mutations even if ACTH deficiency is not apparent at first evaluation. The patient's elder brother and sister were of normal adult height and had normal baseline hormonal levels. Even though this report is not the first description of the wide phenotypic spectrum in *LHX4* mutation carriers [13–17], it is noteworthy that *LHX4* mutation carriers can clinically and endocrinologically present as normal, even though the mutation is nonfunctional. The phenotypical variation documented in this study for patients with MPHD with mutations in *LHX4*, including dissimilarity within probands from the same pedigree, is likely partly due to the impact of other genes that are important but have not been recognized in pituitary development.

In summary, we found that only 3.3% of Japanese patients had mutation. *LHX4* mutation carriers exhibit wide phenotypic variability and can present as normal clinically and endocrinologically, even though they had a nonfunctional mutation. Gradual loss of ACTH should be monitored in CH patients with *LHX4* mutations.

Acknowledgments

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Author Contributions

Conceived and designed the experiments: MT SN TH. Performed the experiments: MT. Analyzed the data: MT. Contributed reagents/materials/analysis tools: TI MI NA SN YH YA KM MA. Wrote the paper: MT TH.

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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

Yumi Asakura,[1]* Koji Muroya,[1] Takeshi Sato,[1] Kenji Kurosawa,[2] Gen Nishimura,[3] and Masanori Adachi[1]

[1]Department of Endocrinology and Metabolism, Kanagawa Children's Medical Center, Mutsukawa, Minami-ku, Yokohama-shi, Kanagawa, Japan

[2]Department of Genetics, Kanagawa Children's Medical Center

[3]Department of Pediatric Imaging, Tokyo Metropolitan Children's Medical Center, Musashidai, Fuchu-shi, Tokyo, Japan

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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.

*Correspondence to:
Yumi Asakura, M.D., Department of Endocrinology and Metabolism, Kanagawa Children's Medical Center, 2-138-4 Mutsukawa, Minami-ku, Yokohama-shi, Kanagawa 232-8555, Japan.
E-mail: yumi79@mvb.biglobe.ne.jp

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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.

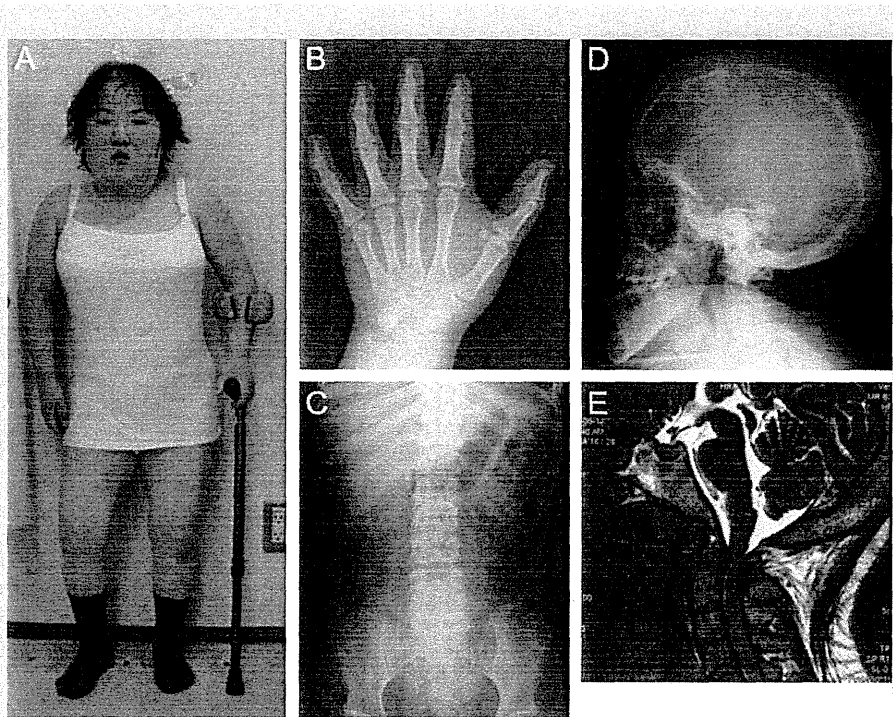

FIG. 1. Clinical manifestations of Myhre syndrome in our patient (A). Note the rhizomelic shortening of the limbs, impressive square body shape, flat wide facies with distinct prognathism, deep-set eyes, short palpebral fissures, and narrow mouth with thin upper lip (A). Skeletal manifestations of the 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 μg/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 *SMAD*4 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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CLINICAL STUDY

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

Masanori Adachi[1,2], Akiko Soneda[1], Yumi Asakura[1], Koji Muroya[1], Yuji Yamagami[2,3] and Fumiki Hirahara[2,4]

[1]*Department of Endocrinology and Metabolism, Kanagawa Children's Medical Center, Mutsukawa 2-138-4, Minami-ku, Yokohama 232-8555, Japan,* [2]*Neonatal Mass-screening Committee, Kanagawa Prefecture Medical Association, Yokohama, Japan,* [3]*Kanagawa Health Service Association, Yokohama, Japan and* [4]*Department of Obstetrics and Gynecology, Yokohama City University School of Medicine, Yokohama, Japan*

(Correspondence should be addressed to M Adachi at Department of Endocrinology and Metabolism, Kanagawa Children's Medical Center; Email: madachi@mars.sannet.ne.jp)

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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