7 Sykiotis GP, Plummer L, Hughes VA, Au M, Durrani S, Nayak-Young S, Dwyer AA, Quinton R, Hall JE, Gusella JF, Seminara SB, Crowley WF Jr, Pitteloud N: Oligogenic basis of isolated gonadotropin-releasing hormone deficiency. Proc Natl Acad Sci USA 2010;107:15140–15144.
8 Trarbach EB, Abreu AP, Silveira LF, Garmes HM, Baptista MT, Teles MG, Costa EM, Mohammadi M, Pitteloud N, Mendonca BB, Latronico AC: Nonsense mutations in FGF8 gene causing different degrees of human gonadotropin-releasing deficiency. J Clin Endocrinol Metab 2010;95:3491–3496.
9 Arauz RF, Solomon BD, Pineda-Alvarez DE, Gropman AL, Parsons JA, Roessler E, Muenke M: A hypomorphic allele in the FGF8 gene contributes to holoprosencephaly and is allelic to gonadotropin-releasing hormone deficiency in humans. Mol Syndromol 2010;1:59–66.
10 Topaloglu AK, Kotan LD: Molecular causes of hypogonadotropic hypogonadism. Curr Opin Obstet Gynecol 2010;22:264–270.
11 Beate K, Joseph N, Nicolas de R, Wolfram K: Genetics of isolated hypogonadotropic hypogonadism: role of GnRH receptor and other genes. Int J Endocrinol 2012;2012:147893.
12 Haraguchi R, Suzuki K, Murakami R, Sakai M, Kamikawa M, Kengaku M, Sekine K, Kawano H, Kato S, Ueno N, Yamada G: Molecular analysis of external genitalia formation: the role of fibroblast growth factor (Fgf) genes during genital tubercle formation. Development 2000;127:2471–2479.
13 Kuzmiak HA, Maquat LE: Applying nonsense-mediated mRNA decay research to the clinic: progress and challenges. Trends Mol Med 2006;12:306–316.
14 Melmed S, Kleinberg D, Ho K: Pituitary physiology and diagnostic evaluation; in Melmed S, Polonsky KS, Larson PR, Kronenberg HM (eds): Williams Textbook of Endocrinology, ed 12. Philadelphia, Saunders, 2011, pp 175–228.
15 Park EJ, Watanabe Y, Smyth G, Miyagawa-Tomita S, Meyers E, Klingensmith J, Camenisch T, Buckingham M, Moon AM: An FGF autocrine loop initiated in second heart field mesoderm regulates morphogenesis at the arterial pole of the heart. Development 2008;135:3599–3610.
16 Wendl T, Adzic D, Schoenebeck JJ, Scholpp S, Brand M, Yelon D, Rohr KB: Early developmental specification of the thyroid gland depends on han-expressing surrounding tissue and on FGF signals. Development 2007;134:2871–2879.
17 Watanabe Y, Miyagawa-Tomita S, Vincent SD, Kelly RG, Moon AM, Buckingham ME: Role of mesodermal FGF8 and FGF10 overlaps in the development of the arterial pole of the heart and pharyngeal arch arteries. Circ Res 2010;106:495–503.
18 Lania G, Zhang Z, Huynh T, Caprio C, Moon AM, Vitelli F, Baldini A: Early thyroid development requires a Tbx1-Fgf8 pathway. Dev Biol 2009;328:109–117.
19 Abu-Issa R, Smyth G, Smoak I, Yamamura K, Meyers EN: Fgf8 is required for pharyngeal arch and cardiovascular development in the mouse. Development 2002;129:4613–4625.
20 Meyers EN, Lewandoski M, Martin GR: An Fgf8 mutant allelic series generated by Cre- and Flp-mediated recombination. Nat Genet 1998;18:136–141.

CLINICAL REPORT

AMERICAN JOURNAL OF medical genetics PART A

# Long-Term Follow-Up Study for a Patient with Floating–Harbor Syndrome Due to a Hotspot *SRCAP* Mutation

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Manuscript Received: 27 May 2013; Manuscript Accepted: 9 September 2013

Floating–Harbor syndrome (FHS) is a rare autosomal dominant disorder characterized by short stature, skeletal malformations, speech delay, and dysmorphic facial appearance. Recently, mutations in *SRCAP* encoding a coactivator for cAMP-response element binding protein (CREB)-binding protein have been identified in small number of patients with FHS. Here, we report on long-term follow-up data of a male patient with a *SRCAP* mutation. The patient presented with mild hypothyroidism and renal hypouricemia, in addition to several FHS-compatible features including growth impairment, cognitive disability, facial dysmorphisms, and hypertension. He showed delayed bone age from infancy to 9 years of age and markedly accelerated bone age with the formation of cone-shaped epiphyses and early epiphysial fusions after the onset of puberty. His pubertal sexual development was almost age appropriate. Two-year treatment with growth hormone (GH) did not significantly improve the growth velocity. Molecular analysis identified a de novo heterozygous nonsense mutation (p.R2444X) in the last exon of *SRCAP*, which has been most common mutation detected in patients from other ethnic groups. These results indicate that perturbed skeletal maturation from infancy through adolescence is a characteristic feature in patients with *SRCAP* mutations. Furthermore, our data imply that GH therapy exerted only a marginal effect on the growth of this patient, and that renal hypouricemia may be a novel complication of FHS. 

**Key words:** Floating–Harbor syndrome; growth; puberty; mutation; *SRCAP*; short stature

**How to Cite this Article:**
Nagasaki K, Asami T, Sato H, Ogawa Y, Kikuchi T, Saitoh A, Ogata T, Fukami M. 2013. Long-term follow-up study for a patient with Floating–Harbor syndrome due to a hotspot *SRCAP* mutation.
Am J Med Genet Part A 9999:1–5.

## INTRODUCTION

Floating–Harbor syndrome (FHS; OMIM#136140) is a rare autosomal dominant disorder characterized by short stature, skeletal malformations, speech delay, and dysmorphic facial appearance [Pelletier and Feingold, 1973; Leisti et al., 1975]. Recent studies have identified 14 mutations in *SRCAP* in a total of 59 patients with FHS [Hood et al., 2012; Reschen et al., 2012; Le Goff et al., 2013; Nikkel et al., 2013]. *SRCAP* consists of 34 exons and encodes an SNF2-related cAMP-response element binding protein (CREB)-binding protein (CREBBP) activator protein with a helicase-SANT-associated domain, dimerization domain, SNF2-like ATPase, CREBBP-binding site, and three C-terminal AT-hook DNA-binding motifs [Johnston et al., 1999]. All the *SRCAP* mutations identified to date were nonsense and frameshift mutations in the last exon (exon 34)

Conflict of interest: none.
Grant sponsor: Ministry of Education, Culture, Sports, Science and Technology (MEXT); Grant number: 22132004; Grant sponsor: Japan Society for the Promotion of Science (JSPS); Grant number: 23390249; Grant sponsor: Takeda Science Foundation and from National Center for Child Health and Development.
Abbreviations: CBP, CREB-binding protein; FHS, Floating–Harbor syndrome.
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Article first published online in Wiley Online Library (wileyonlinelibrary.com): 00 Month 2013
DOI 10.1002/ajmg.a.36314

that presumably escape nonsense-mediated mRNA decay, indicating a dominant-negative effect of the truncated proteins [Monroy et al., 2001; Hood et al., 2012]. Since *SRCAP* mutations have not been identified in a substantial proportion of patients, FHS seems to be a genetically heterogeneous condition [Le Goff et al., 2013].

Given the small number of patients reported so far, further studies are necessary to clarify the phenotypes of patients with *SRCAP* mutations. In particular, the longitudinal growth pattern has been poorly investigated in such patients, although previous studies have shown that severe short stature and markedly delayed bone age in childhood are consistent features in patients with FHS [White et al., 2010]. Here, we report on the long-term follow-up data of a Japanese patient with a hotspot mutation in *SRCAP*. The results provide novel information on the clinical consequences of *SRCAP* mutations.

## CLINICAL REPORT

This male patient was born to nonconsanguineous Japanese parents at 38 weeks of gestation. His parents and sister were clinically normal. At birth, his body length was 43 cm (−2.8 SD) and weight was 2.27 kg (−1.7 SD). Neonatal mass-screening tests showed an elevated thyrotropin level. Endocrine evaluations at 38 days of age showed a slightly elevated serum TSH level (12.0 mIU/L, reference range <10.0 mIU/L) together with a mildly decreased free thyroxine level (1.1 ng/dl, reference range 1.2–2.0 ng/dl). Ultrasonography delineated a normal thyroid gland. Roentgenograms showed the absence of the epiphysis of the distal femur, which is indicative of hypothyroidism. Therefore, the patient was diagnosed as having congenital hypothyroidism and treated with levothyroxine (7 μg/kg/day). Subsequently, TSH levels remained within the normal

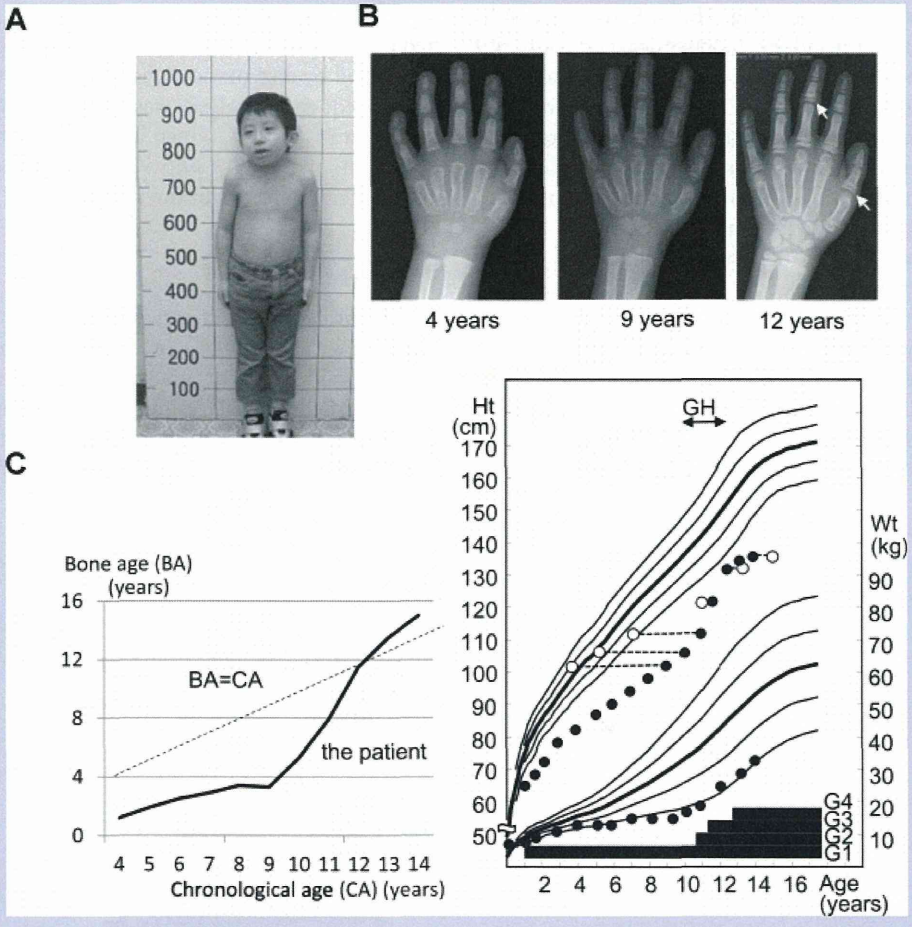


FIG. 1. Clinical findings of the patient. A: Photographs at 8 years of age: Physical findings include proportional short stature, short neck, prominent nose, wide mouth, and short palpebral fissures. B: Roentogenographs of the left hand. Shortening of almost all tubular bones at all ages, delayed bone age before puberty (at 4 and 9 years of age), and cone-shaped epiphyses (arrows) and early epiphyseal fusions after the onset of puberty (at 12 years of age) are shown. C: Growth pattern. Left panel: the solid line indicates the bone age of the patient. The dotted line depicts a reference for age-appropriate skeletal maturation. Right panel: actual height (black circles), bone age (white circles), and pubertal stages (black boxes) are shown. The patient received growth hormone therapy (GH) from 10 to 12 years of age. G1–4; Genital development of Tanner stage 1–4.

range under levothyroxine supplementation. His gross motor milestones were moderately delayed from infancy (sitting alone without support at 7 months of age and walking alone at 21 months of age), while language development was severely retarded (only a few words spoken at 5 years of age). He had a mild chronic constipation with abdominal distension during infancy.

At 8 years of age, the patient underwent detailed clinical evaluation for severe short stature. He showed proportionate short stature with a height of 99.6 cm (−4.9 SD) and weight of 13.9 kg (−2.4 SD). He exhibited dysmorphic facial appearance, including a wide mouth, thin vermilion of the upper lip, short palpebral fissures, malocclusion with small teeth, and low-set and posteriorly rotated ears (Fig. 1A). The ocular findings included amblyopia due to strabismus and astigma that required spectacles. Physical examinations revealed high-pitched voice, short neck, kyphosis, and small hands and feet. He had a WISC-III verbal score of 43, a performance score of 68, and full scale score of 50 indicating cognitive disability. Hand roentgenograms delineated brachydactyly and severely retarded bone age (Fig. 1B,C). Ultrasonography detected no abnormality in the kidney. Biochemical and endocrine studies showed no abnormalities except for hypouricemia caused by hyperuricosuria (serum uric acid, 1.7 mg/dl; normal range 3.7–7.5 mg/dl, fractional excretion of uric acid, 18%; normal range 5–15%). Since his TSH and thyroid hormone levels remained normal after a four-week discontinuation of levothyroxine, levothyroxine supplementation was discontinued at this time point.

From 10 years of age, the patient was treated with growth hormone (GH) (0.23 mg/kg/week) for the indication of short stature born small for gestational age. However, this therapy was discontinued after 2 years because of a poor response (growth velocity, 4.1 cm/year before GH treatment and 5.0 cm/year at 1 year after treatment). He exhibited almost age-matched sexual maturation with testicular enlargement from 11 years of age, pubic hair from 12 years of age, and voice deepens from 13 years of age. Bone age development markedly accelerated from 9 years of age and reached a nearly adult level when the patient was 13 years of age (Fig. 1C). Hand roentgenograms at 12 years of age showed cone-shaped epiphyses of the 2nd and 3rd middle phalanges and the first basal phalanx, and early epiphyseal fusions (Fig. 1C). His nearly final height at 14 years of age was 137 cm (−3.6 SD). From 13 years of age, he developed hypertension (blood pressure, 133/94 mmHg). He attended a special educational program in a junior high school and had mild behavior and emotional problems.

## MOLECULAR ANALYSIS

This study was approved by the Institutional Review Board Committees at the National Center for Child Health and Development and performed after obtaining written informed consent from the parents. G-banding analysis showed a normal 46,XY karyotype in the patient. Thus, direct sequence analysis was carried out for exon 34 of *SRCAP* using leukocyte genomic DNA samples. A previously reported nonsense mutation (c.7330C>T, p.R2444X) was identified in the patient but not in the parents (Fig. 2A). The results were confirmed by enzymatic digestion of the PCR products (Fig. 2B). Direct sequence analysis was also performed for the p.W258X mutation in *URAT1*, which accounts for the majority of genetic defects in Japanese patients with renal hypouricemia. This mutation was not detected in the patient.

## DISCUSSION

We identified a de novo heterozygous mutation in *SRCAP* (c.7330C>T, p.R2444X) in a Japanese male patient with FHS. The clinical features of this patient including short stature, developmental delay, facial dysmorphism, and hypertension were comparable with those of previously reported patients with *SRCAP* mutations (Table I). Furthermore, hypothyroidism has recently

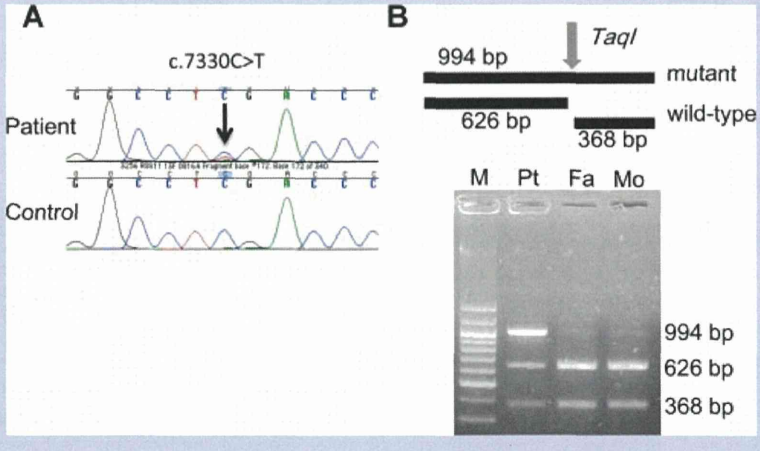


FIG. 2. Molecular analysis. A: Chromatogram indicating the *SRCAP* mutation (c.7330C>T; p.R2444X). The arrows indicate the mutated nucleotide. B: Enzymatic digestion of PCR products. The wild-type PCR product (994 bp) is digested into 626 and 368 bp fragments by the restriction enzyme *TaqI*, whereas the mutant PCR product remains undigested. M, marker; Pt, patient; Fa, father; and Mo, mother.

**TABLE I. Clinical Features in Patients With Genetically Proved Floating–Harbor Syndrome**

| Clinical features | Previous cases[a] | Present case |
|---|---|---|
| Short stature | 41/52[b] | Yes |
| Finger malformation | 10/17[b] | Yes |
| Neuropsychological features | | |
| Speech delay | 100%[b,c] | Yes |
| Behavior problem | 9/32[b] | Yes |
| Craniofacial features | | |
| Strabismus | 7/43[b] | Yes |
| Hyperopia | 5/43[b] | Yes |
| Recurrent otitis media | 6/52[b] | No |
| Hearing loss | 9/52[b] | No |
| High pitched voice | 8/11[b] | Yes |
| Small teeth/increased spacing | 13/38[b] | Yes |
| Caries | 6/38[b] | No |
| Cardiac malformation | 3/52[b] | No |
| Gastrointestinal feature | | |
| Motility issue | 13/52[b] | Yes (constipation) |
| Genitourinary features | | |
| Cryptorchidism | 5/24[b] | No |
| Renal tract anomaly | 7/U[b] | No |
| Hypouricemia | N.D. | Yes |
| Hormonal abnormalities | | |
| Precocious puberty | 1/3[d] | No |
| Hypothyroidism | 2/52[b] | Yes (transient) |
| Hypertension | 3/U[c,e] | Yes |

ND, not described; U, denominator unknown.
[a]The denominators indicate the number of patients examined for the presence or absence of each feature, and the numerators represent the number of patients assessed to be positive for that feature.
[b]Reported by Nikkel et al. [2013].
[c]Reported by Reschen et al. [2012].
[d]Reported by Le Goff et al. [2013].
[e]The clinically confirmed patients are included in the numerator.

been described in two cases with such mutations [Nikkel et al., 2013]. These data support the notion that FHS is caused by protein-truncating mutations in the last exon of *SRCAP*. The p.R2444X is the most common mutation detected in patients from various ethnic groups with FHS to date [Nikkel et al., 2013].

We were able to follow up this patient from birth to nearly final height. Three aspects are noteworthy. First, his bone age was severely retarded from infancy to 9 years of age and markedly accelerated subsequently. These findings are consistent with those of previous studies showing significantly delayed bone age in patients <6 years of age and variably delayed or normal bone age in patients between 6 and 12 years of age [White et al., 2010]. Since our patient manifested almost age-appropriate sexual development, his accelerated skeletal maturation seems to be ascribed to perturbed proliferation or differentiation of chondrocytes rather than sexual precocity. Indeed, the patient manifested brachydactyly and early epiphyseal fusions with cone-shaped epiphyses, which indicate dysregulated chondrocyte maturation. Unique growth pattern of this patient may reflect abnormal chondrocyte development, because elongation of epiphyseal growth plates primarily depends on proliferation and differentiation of chondrocytes [Wuelling and Vortkamp, 2011]. SRCAP, by virtue of its ability to interact with CREBBP, is supposed to be involved in several signaling pathways including cAMP-mediated G protein-coupled receptor signaling [Monroy et al., 2001]. Because congenital disorders of the cAMP-mediated signaling pathway such as pseudohypoparathyroidism type Ia and acrodysostosis are known to result in various skeletal malformations including early epiphyseal fusions with cone-shaped epiphyses and brachydactyly [Bastepe et al., 2000], skeletal abnormalities in patients with *SRCAP* mutations may also be caused by impaired cAMP-mediated signaling. However, because precocious puberty has been described in a few patients with FHS, relatively early sexual maturation may underlie rapid bone age progression in some patients [Stagi et al., 2007; White et al., 2010; Nikkel et al., 2013].

Second, the impaired growth of our patient was not ameliorated after 2-year GH treatment. Furthermore, we cannot exclude the possibility that GH treatment further accelerated bone maturation in our patient, because his bone age significantly progressed in the period when he received GH. These results are inconsistent with those of previous reports. Wieczorek et al. have reported a female patient with normal GH levels who achieved a normal height (−0.9 SD) after 3-year GH treatment from 5 years of age, although the final height of this patient has not been described [Wieczorek et al., 2001]. Similarly, Stagi et al. [2007] have described a female patient with neurosecretory GH dysfunction and precocious puberty who reached a normal adult height (−1.20 SD) after treatment with GH and gonadotrophin-releasing hormone analog. Thus, further studies are required to clarify the effectiveness of pharmacological interventions for short stature in patients with *SRCAP* mutations.

Third, our patient presented with renal hypouricemia, which has not been reported in patients with FHS. Renal hypouricemia is known as an inherited disorder characterized by impaired renal uric acid reabsorption and low serum uric acid levels that may cause exercise-induced acute renal failure and nephrolithiasis. We confirmed that the patient did not have the p.W258X mutation in *URTA1*, which accounts for the majority of genetic defects in Japanese patients with renal hypouricemia [Ichida et al., 2008]. Since several renal abnormalities such as nephrocalcinosis, polycystic kidney and hematuria have been reported in patients with FHS [Reschen et al., 2012], renal hypouricemia may also be associated with the *SRCAP* mutation. Thus, it remains to be elucidated whether renal hypouricemia is a common manifestation in patients with *SRCAP* mutations.

In summary, the present study provides further information on unique phenotypes resulting from *SRCAP* mutations. Further studies will permit better clarification of the phenotypic spectrum in patients with this condition.

## ACKNOWLEDGMENTS

This work was supported by the Grant-in-Aid for Scientific Research on Innovative Areas (22132004) from the Ministry of

Education, Culture, Sports, Science and Technology (MEXT), by the Grant-in-Aid for Scientific Research (B) (23390249) from the Japan Society for the Promotion of Science (JSPS), by Grants from Takeda Science Foundation and from National Center for Child Health and Development.

## REFERENCES

Bastepe M, Jüppner H, 2000. Pseudohypoparathyroidism. New insights into an old disease. Endocrinol Metab Clin North Am 29:569–589.

Hood RL, Lines MA, Nikkel SM, Schwartzentruber J, Beaulieu C, Nowaczyk MJ, Allanson J, Kim CA, Wieczorek D, Moilanen JS, Lacombe D, Gillessen-Kaesbach G, Whiteford ML, Quaio CR, Gomy I, Bertola DR, Albrecht B, Platzer K, McGillivray G, Zou R, McLeod DR, Chudley AE, Chodirker BN, Marcadier J, FORGE Canada Consortium, Majewski J, Bulman DE, White SM, Boycott KM, 2012. Mutations in SRCAP, encoding SNF2-related CREBBP activator protein, cause Floating–Harbor syndrome. Am J Hum Genet 90:308–313.

Ichida K, Hosoyamada M, Kamatani N, Kamitsuji S, Hisatome I, Shibasaki T, Hosoya T. 2008. Age and origin of the G774A mutation in SLC22A12 causing renal hypouricemia in Japanese. Clin Genet 74:243–251.

Johnston H, Kneer J, Chackalaparampil I, Yaciuk P, Chrivia J. 1999. Identification of a novel SNF2/SWI2 protein family member, SRCAP, which interacts with CREB-binding protein. J Biol Chem 274:16370–16376.

Le Goff C, Mahaut C, Bottani A, Doray B, Goldenberg A, Moncla A, Odent S, Nitschke P, Munnich A, Faivre L, Cormier-Daire V. 2013. Not all Floating–Harbor syndrome cases are due to mutations in exon 34 of SRCAP. Hum Mutat 34:88–892.

Leisti J, Hollister DW, Rimoin DL. 1975. The Floating–Harbor syndrome. Birth Defects Orig Artic Ser 11:305.

Monroy MA, Ruhl DD, Xu X, Granner DK, Yaciuk P, Chrivia JC. 2001. Regulation of cAMP-responsive element-binding protein-mediated transcription by the SNF2/SWI-related protein, SRCAP. J Biol Chem 276:40721–40726.

Nikkel SM, Dauber A, de Munnik S, Connolly M, Hood RL, Caluseriu O, Hurst J, Kini U, Nowaczyk MJ, Afenjar A, Albrecht B, Allanson JE, Balestri P, Ben-Omran T, Brancati F, Cordeiro I, da Cunha BS, Delaney LA, Destrée A, Fitzpatrick D, Forzano F, Ghali N, Gillies G, Harwood K, Hendriks YM, Héron D, Hoischen A, Honey EM, Hoefsloot LH, Ibrahim J, Jacob CM, Kant SG, Kim CA, Kirk EP, Knoers NV, Lacombe D, Lee C, Lo IF, Lucas LS, Mari F, Mericq V, Moilanen JS, Møller ST, Moortgat S, Pilz DT, Pope K, Price S, Renieri A, Sá J, Schoots J, Silveira EL, Simon ME, Slavotinek A, Temple IK, van der Burgt I, de Vries BB, Weisfeld-Adams JD, Whiteford ML, Wierczorek D, Wit JM, Yee CF, Beaulieu CL, White SM, Bulman DE, Bongers E, Brunner H, Feingold M, Boycott KM. 2013. The phenotype of Floating–Harbor syndrome: Clinical characterization of 52 individuals with mutations in exon 34 of SRCAP. Orphanet J Rare Dis 8:63.

Pelletier G, Feingold M. 1973. Case report 1. In: Bergsma D, editor. Syndrome identification. White Plains, NY: National Foundation-March of Dimes. pp 8–9.

Reschen M, Kini U, Hood RL, Boycott KM, Hurst J, O'Callaghan CA. 2012. Floating–Harbor syndrome and polycystic kidneys associated with SRCAP mutation. Am J Med Genet Part A 158A:3196–3200.

Stagi S, Galluzzi F, Bindi G, Lapi E, Cecchi C, Salti R, Chiarelli F. 2007. Precocious puberty in a girl with Floating–Harbor syndrome. J Pediatr Endocrinol Metab 20:1333–1337.

White SM, Morgan A, Da Costa A, Lacombe D, Knight SJ, Houlston R, Whiteford ML, Newbury-Ecob RA, Hurst JA. 2010. The phenotype of Floating–Harbor syndrome in 10 patients. Am J Med Genet Part A 152A:821–829.

Wieczorek D, Wüsthof A, Harms E, Meinecke P. 2001. Floating–Harbor syndrome in two unrelated girls: Mild short stature in one patient and effective growth hormone therapy in the other. Am J Med Genet 104: 47–52.

Wuelling M, Vortkamp A. 2011. Chondrocyte proliferation and differentiation. Endocr Dev 21:1–11.

ORIGINAL ARTICLE: GENETICS

# Genome-wide copy number analysis and systematic mutation screening in 58 patients with hypogonadotropic hypogonadism

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**Objective:** To clarify the molecular basis of hypogonadotropic hypogonadism (HH).
**Design:** Genome-wide copy number analysis by array-based comparative genomic hybridization and systematic mutation screening of 29 known causative genes by next-generation sequencing, followed by in silico functional assessment and messenger RNA/DNA analyses of the mutants/variants.
**Setting:** Research institute.
**Patient(s):** Fifty-eight patients with isolated HH (IHH), combined pituitary hormone deficiency (CPHD), and syndromic HH.
**Intervention(s):** None.
**Main Outcome Measure(s):** Frequency and character of molecular abnormalities.
**Result(s):** Pathogenic defects were identified in 14 patients with various types of HH, although oligogenicity was not evident in this patient group. As rare abnormalities, we identified a submicroscopic deletion involving *FGFR1* and an *SOX3* polyalanine deletion in patients with IHH, and a *WDR11* splice site mutation in a patient with CPHD. No disease-associated polymorphism was detected in the 58 patients.
**Conclusion(s):** The present study provides further evidence that mutations and deletions in the known causative genes play a relatively minor role in the etiology of HH and that submicroscopic rearrangements encompassing *FGFR1* can lead to IHH as a sole recognizable clinical feature. Furthermore, the results indicate for the first time that polyalanine deletions in *SOX3* and mutations in *WDR11* constitute rare genetic causes of IHH and CPHD, respectively. (Fertil Steril® 2014;■:■–■. ©2014 by American Society for Reproductive Medicine.)
**Key Words:** *FGFR1*, genomic rearrangements, gonadotropin deficiency, mutation, *SOX3*, *WDR11*

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Received April 4, 2014; revised May 28, 2014; accepted June 11, 2014.
Y.I. has nothing to disclose. E.S. has nothing to disclose. S. Kanzaki has nothing to disclose. S.Y. has nothing to disclose. S. Kinjo has nothing to disclose. M.I. has nothing to disclose. T.M. has nothing to disclose. S.S. has nothing to disclose. R.H. has nothing to disclose. N.S. has nothing to disclose. K.N. has nothing to disclose. K.H. has nothing to disclose. A.U. has nothing to disclose. T.O. has nothing to disclose. Y.Y. has nothing to disclose. M.F. has nothing to disclose.
Supported by the Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology; from the Japan Society for the Promotion of Science (grant 22132004-A01, Tokyo, Japan); by the grants from the Ministry of Health, Labor and Welfare, from National Center for Child Health and Development (grant 23A-1, 24-7, Tokyo, Japan); and from Takeda foundation.
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Fertility and Sterility® Vol. ■, No. ■, ■ 2014 0015-0282/$36.00

http://dx.doi.org/10.1016/j.fertnstert.2014.06.017

Hypogonadotropic hypogonadism (HH) is a clinically and genetically heterogeneous condition that occurs as an isolated hormonal defect (isolated HH, IHH) or in combination with other pituitary hormone deficiencies (combined pituitary hormone deficiency, CPHD) (1). Hypogonadotropic hypogonadism can also take place as a component of congenital syndromes such as Kallmann syndrome (KS), septo-optic dysplasia, and CHARGE syndrome (1–4). Hypogonadotropic hypogonadism affects men and women, although the prevalence is significantly higher in men than in women (5). Monoallelic, biallelic, and oligogenic mutations in more than 15 genes including *FGFR1*, *PROKR2*, *KAL1*, and *CHD7* have been identified in patients with HH (1, 6–11). In addition, missense mutations in *WDR11* and polyalanine deletions in *SOX3* have recently been described as rare causes of IHH/KS and CPHD, respectively (12–16). Because of the highly heterogeneous genetic basis of HH, there have been few reports of systematic mutation analyses in patients with this condition (9–11, 17). Furthermore, genome-wide copy number analyses have rarely been performed for patients with HH, although submicroscopic deletions involving *KAL1*, *FGFR1*, *GNRH1*, and *KISS1R* have been identified in a small number of patients (17–25). Thus, the current understanding of the etiology of HH remains fragmentary.

Recent advances in molecular technology including array-based comparative genomic hybridization and next-generation sequencing have enabled researchers to perform genome-wide copy number analysis and high-throughput mutation screening for multiple samples. Here, using comparative genomic hybridization and next-generation sequencing, we analyzed samples from 58 patients with various types of HH. Our results provide novel information about the molecular abnormalities underlying this condition.

## MATERIALS AND METHODS

### Patients

We studied 58 unrelated Japanese patients with HH (34 male and 24 female subjects, aged 0–56 years). The patient group included patients 1–10 and 35–45 with normosmic IHH (nIHH), patients 11–25 and 46–51 with KS, patients 26–28 and 52 with CPHD, patients 29 and 53–55 with CHARGE syndrome, and patients 30 and 31 with septo-optic dysplasia. Patients 32–34 and 56–58 were IHH patients who did not undergo quantitative assessment of olfactory function; these patients should have either nIHH or KS. Clinical data of the patients are shown in Supplemental Table 1 (available online). One female subject (patient 45) mothered a child by IVF after treatment with hMG, whereas fertility was unknown for the remaining 57 patients. Patients with cytogenetically detectable chromosomal abnormalities were excluded from the study. Patient 13 has been reported previously (26).

### Copy Number Analysis

This study was approved by the Institutional Review Board Committee at the National Center for Child Health and Development. After obtaining written informed consent, genomic DNA was extracted from peripheral leukocytes of the patients. We examined copy number alterations in the entire genome using an array-based catalog comparative genomic hybridization (4 × 180 k format, catalog number G4449A; Agilent Technologies). In the present study, we focused on copy number changes involving the known causative genes and those involving genomic intervals more than 1.5 Mb, which have a higher probability of being associated with disease phenotypes (27). To exclude benign copy number variants, we referred to the database of genomic variants (http://projects.tcag.ca/variation/).

### Mutation Analysis

Mutation analysis was carried out for the coding regions of 29 known HH causative genes, namely, *CHD7*, *FGF8*, *FGFR1*, *FSHB*, *GNRH1*, *GNRHR*, *HESX1*, *HS6ST1*, *KAL1*, *KISS1*, *KISS1R*, *LEP*, *LEPR*, *LHB*, *LHX3*, *LHX4*, *NELF*, *NR0B1*, *OTX2*, *POU1F1*, *PROK2*, *PROKR2*, *PROP1*, *SEMA3A*, *SOX2*, *SOX3*, *TAC3*, *TACR3*, and *WDR11* (8, 28–30). Sequences were determined by the Haloplex system (Agilent Technologies) on a MiSeq sequencer (Illumina). The average read depth in target regions was 322.9. Nucleotide changes were identified using the Surecall system (Agilent Technologies) and SAMtools v0.1.17 software (http://samtools.sourceforge.net/). In the present study, we focused on nonsynonymous substitutions and splice site mutations. Single nucleotide polymorphisms (SNPs) of allele frequency >1.0% in the Japanese general population (dbSNP, http://www.ncbi.nlm.nih.gov/) and nucleotide changes identified in our control samples (unaffected Japanese individuals, n = 16) were excluded from further study. Nucleotide substitutions identified by next-generation sequencing were confirmed by Sanger sequencing. Primer sequences are available upon request.

The functional consequences of missense substitutions were predicted by in silico analysis using PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/). We classified frameshift and nonsense mutations as damaging mutations. We referred to previous articles and the human gene mutation database (http://www.hgmd.cf.ac.uk/ac/index.php) to identify mutations that have been associated with HH. For one patient with a nucleotide change in the consensus sequence at a splice acceptor site of *WDR11*, we performed reverse transcription-polymerase chain reaction (PCR) analysis using lymphoblastoid messenger RNA and a primer pair, 5′-GGGCTGGCAAGGTTTAATTG-3′ and 5′-GCTTGGATGGGGAGAAGTCT-3′. The protein structure of the mutant WDR11 was predicted using the WD40 repeat protein structure predictor (http://wu.scbb.pkusz.edu.cn/wdsp/index.php) (31).

The nucleotide alterations identified in this study were categorized into three groups: pathogenic mutations (mutations previously associated with HH or hitherto unreported mutations that were assessed as "probably damaging" by in silico analyses), apparently benign variants (hitherto unreported substitutions assessed as "benign"), and rare polymorphisms (variants of allele frequency <1.0% in general population). To determine whether the rare polymorphisms are associated with disease risk, allele frequencies in the

**TABLE 1**

**Copy number alterations and nucleotide changes identified in the present study.**

| Case | Sex | Clinical diagnosis | Pathogenic mutation[a] | Apparently benign variant[a] | Rare polymorphism[b] |
|---|---|---|---|---|---|
| 1 | M | nIHH | *FGFR1* deletion | ***NR0B1* p.A127T** | |
| 2 | M | nIHH | ***SOX3* p.A234_242del9A** | | |
| 3 | M | nIHH | *PROKR2* p.D42insED | | *PROKR2* p.W178S |
| 4 | M | nIHH | | *CHD7* p.C2187Y | |
| 5 | M | nIHH | | | *LHB* p.Y57H |
| 11 | M | KS | ***KAL1* g.IVS3+2 T>C** | | |
| 12 | M | KS | *CHD7* p.G998E | *CHD7* p.T2227S | |
| 13 | M | KS | *FGF8* p.S192fsX204 | *LHX4* p.P17S | |
| 14 | M | KS | | | *LHB* p.R88W |
| 15 | M | KS | *CHD7* p.F1352S | | |
| 16 | M | KS | | | *LHX3* p.R208C |
| 19 | M | KS | *FGFR1* p.V102I | | |
| 20 | M | KS | *CHD7* p.R2065C | | |
| 26 | M | CPHD | *WDR11* g.IVS3-2 A>G | | |
| 30 | M | SOD | | | *WDR11* p.A1076T |
| 32 | M | IHH | | *CHD7* p.A2714V | |
| 35 | F | nIHH | | | *PROKR2* p.Y113H |
| 36 | F | nIHH | | *NR0B1* p.A127T | |
| 37 | F | nIHH | | | *LHB* p.Y57H |
| 38 | F | nIHH | | | *LHB* p.Y57H |
| 45 | F | nIHH | *FGFR1* p.S107T | | |
| 46 | F | KS | *FGFR1* p.R570W | | |
| 53 | F | CHARGE | *CHD7* p.K645fsX711 | | *LHX3* p.A322T |
| 54 | F | CHARGE | *CHD7* p.R987X | | |

*Note:* Deletions and nucleotide changes were identified in 24 of 58 patients. CHARGE = CHARGE syndrome; CPHD = combined pituitary hormone deficiency; F = female; nIHH = normosmic isolated hypogonadotropic hypogonadism; IHH = isolated hypogonadotropic hypogonadism with unknown olfactory function; KS = Kallmann syndrome; M = male; SOD = septo-optic dysplasia.

[a] Mutations shown in bold were identified in a homozygous or hemizygous state, while other mutations/variants/polymorphisms were detected in a heterozygous state.

[b] Polymorphisms with an allele frequency of less than 1.0% are shown.

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patient group and in the general population were analyzed by Fisher's exact probability test. *P* values of less than .05 were considered significant.

## RESULTS

### Copy Number Analysis

A submicroscopic genomic rearrangement at 8p11.22–12 was identified in male case 1 (Table 1, Fig. 1A). This rearrangement comprised a ~5.1-Mb deletion and a ~153-kb amplification. The deletion encompassed all exons of *FGFR1*, in addition to the exons of 29 other genes and pseudogenes that have not been associated with HH (Supplemental Table 2, available online). The amplification included two pseudogenes (*ADAM5* and *ADAM3A*). This amplification has been registered in the SNP database as a frequent copy number variant. Parental samples of patient 1 were not available for genetic testing. No pathogenic copy number alterations were identified in the remaining 57 patients.

### Mutation Analysis

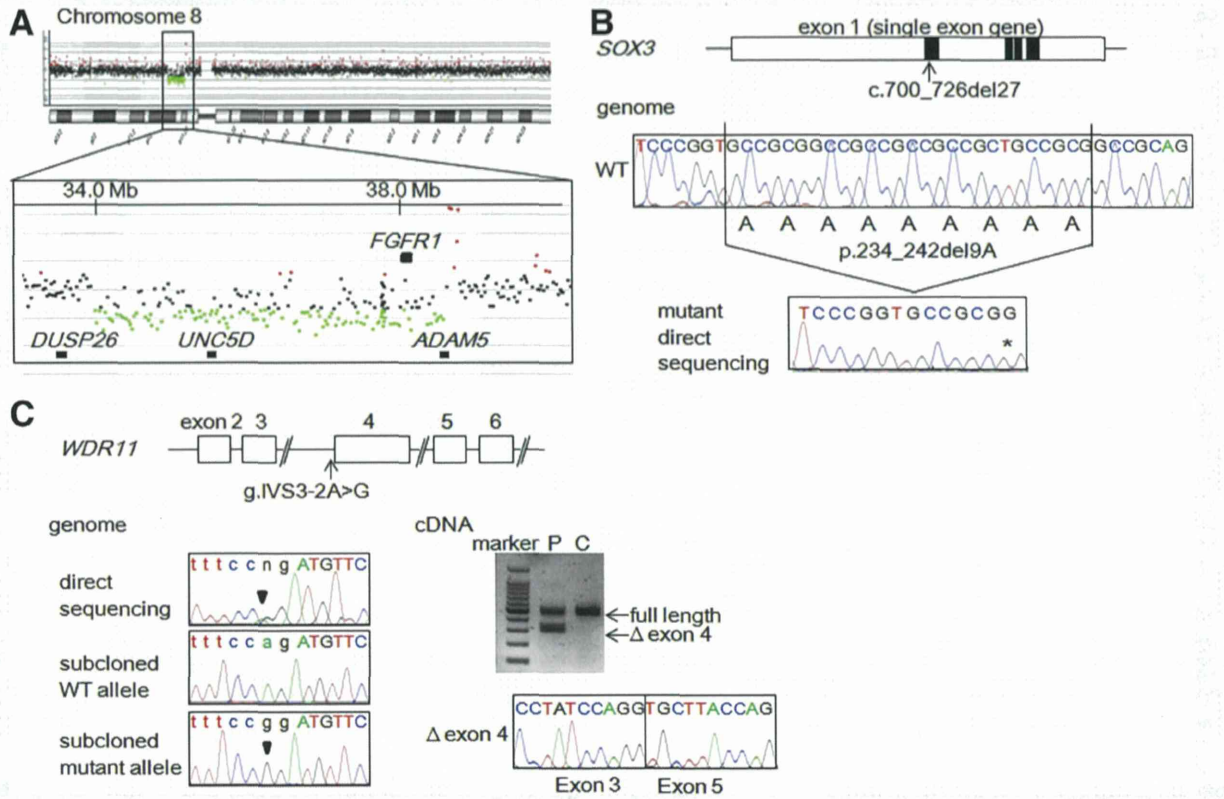
A total of 13 mutations were identified in 9 of 34 male patients and 4 of 24 female patients (Table 1). Mutations in *FGFR1* and *CHD7* accounted for most of the molecular defects. Oligogenicity was not evident in the 58 patients. Rare monogenic mutations were detected in male patients 2 and 26 (Fig. 1B and C). Patient 2 had a hemizygous 27-bp deletion in *SOX3* (c.700_726del27), which resulted in the loss of 9 alanine residues from the first polyalanine tract (p.A234_242del9A). Patient 26 carried a hitherto unreported nucleotide changes at a splice acceptor site of *WDR11* (g.IVS3-2A>G). Reverse transcription-PCR analysis of patient 26 revealed that the mutation led to a deletion of the entire exon 4. The Δexon 4 mutant (c.353_526del174) was predicted to cause a 58-amino-acid deletion and 1-amino-acid insertion (p.D118_L175delinsV), thereby disrupting the functionally important bladed β-propeller structure of the WD40 protein (32). Parental samples of patient 2 were not available. The mother of patient 26 carried the same mutation.

Six apparently benign substitutions and 7 rare polymorphisms were identified in 15 patients (Table 1). The allele frequencies of the seven polymorphisms did not differ between the patient group and the general population, although the frequency of the *LHB* rs371722800 polymorphism in the general population was unknown (Supplemental Table 3, available online).

### Phenotypes of Three Patients with Rare Genetic Defects

The clinical and hormonal findings of patients 1, 2, and 26 are summarized in Table 2. The three patients were men and presented with genital abnormalities and/or lack of pubertal development. Physical examination revealed no additional clinical features in patient 1, short stature in patient 2, and obesity, short stature, and mental retardation in patient 26. Olfactory dysfunction was not apparent in any of the three patients. Brain magnetic resonance imaging revealed normal pituitary in patient 1, and pituitary malformation in patients

**FIGURE 1**




*Rare monogenic defects identified in the present study.* (**A**) *Genomic rearrangement in patient 1. The black, red, and green dots* denote signals in comparative genomic hybridization analyses that indicate the normal, increased (>+0.5), and decreased (<−0.8) copy numbers, respectively. The deletion included all exons of *FGFR1* and exons of an additional 29 genes and pseudogenes. The amplification affected two pseudogenes including *ADAM5*. Genomic positions refer to the human genome database (hg19, build 37). The position of genes around the breakpoints is shown. (**B**) *SOX3* mutation in patient 2. *Top*, genomic position of the mutation. The *black boxes* indicate polyalanine tracts. *Middle and lower*, chromatographs of the mutation. The mutation resulted in a deletion of nine amino acids from the first polyalanine tract. The *asterisk* indicates a common polymorphism. WT = wild type. (**C**) *WDR11* mutation in patient 26. *Top*, genomic position of the mutation. *Left*, chromatographs of the mutation. The mutation (*arrowheads*) affected the splice donor site of exon 4. *Right*, complementary DNA (cDNA) analysis. The mutation resulted in exon skipping. C = control; P = patient.

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2 and 26. Endocrine evaluation indicated grossly normal pituitary function except for HH in patients 1 and 2, and combined deficiencies of LH and GH in patient 26. The mother of patient 26 with the same *WDR11* mutation as the proband allegedly had menstrual irregularity with oligomenorrhea (one cycle of ~6 months), although she had no history of delayed puberty (menarche at age 12 years).

## DISCUSSION

We identified pathogenic molecular defects in 14 of 58 cases with HH. The mutation-positive rate was higher in male than in female patients, reflecting the presence of X-linked mutations in *KAL1* and *SOX3*. Although we identified several other nucleotide alterations that have not been submitted to the SNP database, in silico analyses indicated that most of these substitutions are functionally neutral. Likewise, although rare polymorphisms were detected in nine patients, these SNPs were unlikely to associate with the disease risk, because they were present in the patients at frequencies similar to those of the Japanese general population. However, the allele frequency of *LHB* rs371722800 in the general population needs to be determined to clarify whether this SNP is a susceptibility allele. Our data suggest a relatively minor role of known gene mutations in the development of HH and demonstrate the rarity of oligogenic mutations in Japanese patients.

Three of the 58 patients harbored rare molecular defects. Patient 1 carried a small genomic rearrangement at 8p11.22–12. Chromosomal rearrangements at 8p11.22–12 have been identified in a small number of patients with HH with various complications (19, 21–23). Our findings imply that submicroscopic chromosomal deletions involving *FGFR1* can cause nIHH as a sole recognizable clinical feature. Because the short arm of chromosome 8 is known as a hotspot for chromosomal rearrangements (19, 21–23, 33), such submicroscopic rearrangements may be hidden in a certain fraction of patients with HH. Patient 2 harbored a hemizygous deletion affecting nine alanine residues of the